changes relative to the previous version, the changes indicated are relative to the originally filed version.

The specification has also been amended to correct an inadvertently made, obvious error in the first entry in Table 1 to indicate that Oligo #164 (SEQ ID NO:1) is an antisense oligonucleotide. This error is obvious from the fact that the hybrid, inverted hybrid, and inverted chimeric oligonucleotides are based on this same sequence and the control oligonucleotides are mismatched from this sequence.

Additionally, the specification has also been amended to delete inadvertently inserted data obviously unrelated to this application and to correct the heading of the section containing this data to reflect the data contained therein. Support for the amendment of the heading can be found in one of the parent patents, U.S. Patent Number 5, 969, 117, at Col. 12, line 11, which used the same heading.

Finally, the specification has also been amended to correct the description of Example 10. These changes are obvious from a comparison of Table 1, amended herein, which shows the sequences and SEQ ID NOS, with Figure 1, which shows the results of the experiment.

Thus, no new matter has been introduced by these amendments.

Applicant assumes that all rejections not repeated in the Office Action of June 20, 2001 have been overcome and are withdrawn.

Applicant acknowledges that the formal drawings have been approved by the Draftsperson.

The outstanding rejections are addressed individually below.

1. *Claims 1-33 are enabled by the specification as filed.*

Claims 1-33 stand rejected under 35 U.S.C. § 112, first paragraph, as not being enabled by the specification as filed. Applicants point out that claims 21 and 22 were cancelled in the response filed on March 26, 2001, and thus the rejection of claims 21 and 22 is moot. Applicant respectfully traverses the rejection of claims 1-20 and 23-33.

Claim 1 is directed to a method for inhibiting proliferation of cancer cells comprising administering to the cells a first agent comprising a synthetic, modified oligonucleotide complementary to, and which down-regulates the expression of, nucleic acid encoding protein kinase A subunit RIα, the modified oligonucleotide having from about 15 to about 30 nucleotides and being a hybrid, inverted hybrid, or inverted chimeric oligonucleotide of specific characteristics, administering to the cells a second agent comprising an antibody that binds to EGFR or a chemotoxic agent selected from an enumerated group, wherein the administering steps may be performed simultaneously or sequentially in any order. Other independent claims are further directed to a pharmaceutical composition and a method for treating cancer.

The Office Action states that the specification, while being enabling for inhibiting proliferation of cancer cells *in vitro*, does not reasonably provide enablement for treatment of cancer in a patient *in vivo*, and that the specification does not enable a person skilled in the art to which it pertains to use the invention commensurate in scope with these claims. Applicant maintains that the specification enables the pending claims.

The Office Action further states that the instant application does not provide sufficient enablement for one of skill in the art to practice the full scope of the claimed invention without undue experimentation. (Office Action, page 3). The Office Action cites Crooke (*Antisense Research and Application*, Chapter 1, Basic Principles of Antisense Therapeutics, Springer-Verlag, Berlin, Heidelberg, New York, page 3, 1998) for a variety of factors that influence cellular uptake and distribution of antisense based therapeutics, and states that due to the unpredictability in cellular behavior associated with variations in sequence, length, and modifications of the oligonucleotides encompassed by the present invention, it is likely that the examples comprising the use of the HYB 165 oligonucleotide are not representative of all oligonucleotides encompassed by the claimed invention. (Office Action, page 4) Applicants respectfully disagree.

M.P.E.P § 2164.01 states that 35 U.S.C. § 112, first paragraph, "has been interpreted to require that the claimed invention be enabled so that any person skilled

in the art can make and use the invention without undue experimentation." The same section further states that "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." M.P.E.P § 2164.02 states that "[a]n *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a 'working example' if that example 'correlates' with a disclosed or claimed method invention. . . . In this regard, the issue of 'correlation' is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate." This section further states that a "rigorous or an invariable exact correlation is not required . . . ." M.P.E.P § 2164.03 relates to the relationship of predictability of the art and the enablement requirement; this section states that "what is known in the art provides evidence as to the question of predictability."

Applicant submits that one of ordinary skill in the art would know how to determine effective antisense oligonucleotides without undue experimentation. For example, Milner et al. (*Nature Biotechnology* (1997) **15**:537-541; attached hereto as Appendix A) demonstrates "a combinatorial technique that allows simultaneous assessment of all possible ONs [oligonucleotides] within a given region identifying sequences open to duplex formation. An oligonucleotide 'scanning' array reduces the number of synthesis steps while providing a parallel and exhaustive analysis of all ONs in the region to be targeted." (page 537) This article further states that "those ONs which give high duplex yield on the array proved to be effective antisense agents in in vitro RNase H and translation studies." (page 537) As stated in the abstract, "the arrays provide a simple empirical method of selecting effective antisense oligonucleotides for any RNA target of known sequence." Thus, based on public information, Applicant submits that based on this information, one of ordinary skill in the art would be able to determine effective antisense oligonucleotides without undue experimentation.

Milner et al. also state that "hetroduplex yield on the array correlated well with in vivo and in vitro cell culture antisense activities." (page 540) Milner et al. discusses a reference by Monia et al. (*Nature Medicine* (1996) **2**:668-675; attached hereto as Appendix B), which identified an antisense inhibitor, ISIS 5132. (see page 669) ISIS 5132 was found to display "very potent inhibitory effects" *in vivo* (page 671) and was one of the antisense inhibitors that inhibited expression of C-*raf* in cell culture and *in vivo*. (page 672) This antisense inhibitor was also found to show *in vivo* antitumor effects against two additional tumor cell lines. (page 672) Milner et al. conducted a blind experiment, performing analysis on a scanning array that picked out ISIS 5132 as one of two high-yielding oligonucleotides in a 100 b region around the oligonucleotide. (page 540) Furthermore, as discussed in detail below, many published articles indicate that antisense oligonucleotides have been shown to be effective; therefore, the examples in the specification showing enablement of the invention with respect to one oligonucleotide *in vivo* should be enabling for the other oligonucleotides encompassed by the claims.

Evidence from Galderisi et al. (*J. Cell. Physiol.* (1999) **181**:251-57; attached hereto as Appendix C), indicates that intravenous administration of phosphorothioate oligodeoxynucleotides showed effective and specific antisense inhibition in animal models, that antisense oligodeoxynucleotides have been shown to be effective in preclinical studies, and that some antisense oligodeoxynucleotides have reached clinical trials. The article also teaches that a drug based on antisense technology is now available in the United States. This article provides antisense examples suggesting that such compounds have some therapeutic efficacy, including their use as antiviral agents.

In addition, Agrawal states, at page v of <u>Antisense Therapeutics</u>, (Sudhir Agrawal, ed.) 1996, (cited pages of which are attached hereto as Appendix D), that "[t]he results of preclinical studies using oligodeoxynucleotide phosphorothioates have shown that antisense oligonucleotides have good biological activity, pharmacology, pharmacokinetics, and safety both in vitro and in vivo, and they are currently being

evaluated in human clinical trials for the treatment of viral infections and cancers." Zamecnik (also Appendix D) states at page 6 of the same book that the synthetic antisense oligonucleotide technology displays promising results in cell-free systems, tissue cultures, and animal models and is at early trial points in human testing against HIV, leukemia, Herpes virus, and other diseases.

Craig et al. (*Exp. Opin. Ther. Patents* (1997) 7:1175-1182; attached hereto as Appendix E) teaches at page 1177 that once a modification to the oligonucleotide backbone "is found to confer a favorable characteristic, it can then be used in oligonucleotides having different sequences of nucleosides and, thus, provide utility for the treatment of other diseases" as well as discussing information regarding the patentability of antisense technology.

Furthermore, Monia et al., discussed above, states at page 668 that the authors demonstrate that "treatment of human tumor cells with appropriate phosphorothioate antisense ODNs leads to specific inhibition of *C-raf* gene expression in cell culture and *in vivo*. Moreover, antisense-mediated inhibition of *C-raf* gene expression in human tumor cells results in potent antiproliferative effects in cell culture and potent antitumor activity *in vivo*." As discussed above, this article identified an antisense inhibitor, ISIS 5132, (see page 669), which was found to display "very potent inhibitory effects" *in vivo*. (page 671) This antisense inhibitor was found to show *in vivo* antitumor effects against two additional tumor cell lines. (page 672)

As stated in the Amendment filed on March 26, 2001, Examples 27, 28, and 29 (pages 90-95) as well as Figures 16, 17, and 18 of the instant patent application provide examples and data indicating that the claimed invention does work *in vivo* in an accepted animal model. More specifically, Example 27 indicates that HYB 165 inhibits tumor growth after intraperitoneal or oral administration in mice. The data for this experiment is presented in Figures 16A and 16B. Example 28 indicates that oral HYB 165 cooperatively inhibits tumor growth and increases survival in combination with taxol. Data for this experiment is presented in Figures 17A and 17B. Example 29 indicates that the cooperative antitumor effect of HYB 165 with taxol is accompanied by

inhibition of new vessel formation and growth factor production as well as other results of histochemical analysis. Data for this experiment is presented in the table in Figure 18. Additional support for the *in vivo* use of the methods and pharmaceutical compositions of the invention is found in the description of the figures in the specification at page 20, lines 9-29. Further support for the preferred dosages for the cytotoxic agents and oligonucleotides is found in the specification at page 27, line 15 to page 28, line 14 and page 29, line 8 to page 30, line 10.

Additionally, Example 10 indicates that a single dose of RI_α antisense, hybrid, or inverted hybrid oligonucleotide was tested by injection into the right flank of athymic mice previously innoculated with tumor cells and tumor volumes were obtained. (page 58, line 25 to page 59, line 20), and the results are shown in Figure 1. Thus, it is <u>not</u> only HYB 165 that was shown to work *in vivo*.

The specification also indicates that *in vitro* experiments were performed analyzing, *inter alia*, the effect of inverted hybrid or inverted chimeric structure on oligonucleotide-mediated mitogenicity (page 50, line 16 to page 52, line 6) and to determine the ability of inverted hybrid oligonucleotides and inverted chimeric oligonucleotides to activate RNase H *in vitro* when bound to a complementary RNA molecule (page 56, line 4 to page 57, line 28).

These teachings clearly indicate that the specification enables the claimed invention for both *in vitro* and *in vivo* use by providing supportive data indicating that *in vivo* use of the invention has, in fact, been achieved.

Based on the information provided in the published references described above, the data in the specification indicating that HYB 165 (SEQ ID NO:4) and other oligonucleotide sequences were shown to be operable *in vivo*, and the data showing that other oligonucleotides were operable *in vitro*, Applicant submits that: (1) at least one oligonucleotide works *in vivo* (and in fact more than one oligonucleotide has been shown to work *in vivo*); (2) because there is a correlation between *in vitro* and *in vivo* results, there is a reasonable expectation that antisense oligonucleotides shown to work

in vitro would also be expected to work *in vivo*; (3) it would not require undue experimentation to find other oligonucleotides that would be functional besides HYB 165; and (4) claims only cover operable embodiments, and as stated in M.P.E.P § 2164.03 "even in unpredictable arts [Applicant submits that this art is no longer unpredictable], a disclosure of every operable species is not required." Therefore, applicant submits that the specification enables the scope of the claimed invention.

The Office Action further states that the instant claims read on a method wherein the oligonucleotide of the invention "consists essentially of the nucleotide sequence set forth in SEQ ID NO: 4," and that it is unclear what other sequences are encompassed by this language. Applicant notes that only claims 3, 14, and 24 include such language. Applicant also notes that this language was used in issued claims in a parent application, U.S. Patent No. 5,969,117 (attached hereto as Appendix F). Applicant submits that this language is clear because it indicates that the nucleotide sequence is that sequence set forth in SEQ ID NO: 4.

Therefore, Applicant submits that in view of the foregoing remarks and the references submitted, pending claims 1-20 and 23-33 are enabled by the specification as filed. Accordingly, Applicant respectfully requests that the rejection of these claims under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

CONCLUSIONS

In view of the arguments set forth above, Applicant respectfully submits that the rejections contained in the final Office Action mailed on June 20, 2001, have been overcome, and that the claims are in condition for allowance. If the Examiner believes that any further discussion of this communication would be helpful, she is invited to contact the undersigned at the telephone number provided below.

Applicant encloses herewith a Petition for a One Month Extension of Time pursuant to 37 C.F.R. § 1.136, to respond to the Examiner's Office Action mailed on June

20, 2001. Our deposit account no. 08-0219 is to be charged the $55.00 fee for this purpose.

Applicant also encloses herewith a Supplemental Information Disclosure Statement. Please charge Deposit Account No. 08-0219 the $180.00 fee for this submission.

No other fees are believed to be due in connection with this response. However, please charge any underpayments or credit any overpayments to Deposit Account No. 08-0219.

Respectfully submitted,

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Marked Up Version of Replacement Paragraph in Specification Under 37 C.F.R. §1.121 (b)(1)(iii) Compared to Original Version

Cross-Reference to Related Applications:

This application is a ~~non-provisional~~ continuation-in-part application claiming priority from U.S.S.N. 60/103,098, filed on October 5, 1998, and from U.S.S.N. 09/022,965, filed on February 12, 1998, which is a continuation-in-part application of U.S.S.N. 08/532,979, filed September 22, 1995**, which issued as U.S. Patent No. 5,969,117, which is a continuation-in-part application of U.S.S.N. 08/516,454 filed August 17, 1995, which issued as U.S. Patent No. 5,652,356**.

First entry in Table 1 at page 22:

164	GCG TGC CTC CTC ACT GGC	~~Control~~ **Antisense**	1

Heading at page 36, lines 26-27:

~~Propagation and Quantitation of Cell Lines and Virus Stocks~~

In Vitro Complement Activation Studies

Paragraph at page 58, line 25 to page 59, line 20:

LS-174T human colon carcinoma cells (1×10^6 cells) were inoculated subcutaneously (s.c.) into the left flank of athymic mice. A single dose of RI_α antisense hybrid (Oligo ~~164~~ **165**, SEQ ID NO:4), inverted hybrid (Oligo 166, SEQ ID NO:6), or ~~inverted chimeric~~ **antisense** (Oligo ~~190~~ **164**, SEQ ID NO:1) oligonucleotides or control oligonucleotide (Oligo 169, SEQ ID NO:7); Oligo 168 (SEQ ID NO:5); Oligo 188, (SEQ ID NO:3)~~)~~ as shown in Table 1 (1 mg

per 0.1 ml saline per mouse), or saline (0.1 ml per mouse), was injected s.c. into the right flank of mice when tumor size reached 80 to 100 mg, about 1 week after cell inoculation. Tumor volumes were obtained from daily measurement of the longest and shortest diameters and calculation by the formula, $4/3\pi r^3$ where r = (length + width)/4. At each indicated time, two animals from the control and antisense-treated groups were killed, and tumors were removed and weighed. The results are shown in FIG. 1. These results show that the size of the tumor in the animal treated with the inverted hybrid oligonucleotide 166 having SEQ ID NO:6 was surprisingly smaller from three days after injection onward than the phosphorothioate oligonucleotide 164 having SEQ ID NO:1. That this effect was sequence-specific is also demonstrated in FIG. 1: control oligonucleotide 168 (SEQ ID NO:~~3~~ 5) has little ability to keep tumor size at a minimum relative to the hybrid and inverted hybrid oligonucleotides.

APPENDIX A

Selecting effective antisense reagents on combinatorial oligonucleotide arrays

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An array of 1,938 oligodeoxynucleotides (ONs) ranging in length from monomers to 17-mers was fabricated on the surface of a glass plate and used to measure the potential of oligonucleotide for heteroduplex formation with rabbit β-globin mRNA. The oligonucleotides were complementary to the first 122 bases of mRNA comprising the 5' UTR and bases 1 to 69 of the first exon. Surprisingly few oligonucleotides gave significant heteroduplex yield. Antisense activity, measured in a RNase H assay and by in vitro translation, correlated well with yield of heteroduplex on the array. These results help to explain the variable success that is commonly experienced in the choice of antisense oligonucleotides. For the optimal ON, the concentration required to inhibit translation by 50% was found to be five times less than for any other ON. We find no obvious features in the mRNA sequence or the predicted secondary structure that can explain the variation in heteroduplex yield. However, the arrays provide a simple empirical method of selecting effective antisense oligonucleotides for any RNA target of known sequence.

Keywords: antisense, oligonucleotide, optimization, arrays, secondary structure

Antisense technology holds potential for therapy and for the study of gene function. It also offers an alternative to "knockout" techniques in the characterization of the large number of sequences emerging from genome projects. The concept is appealing: The reagents are readily synthesized, and can be used to down-regulate the expression of any mRNA of known sequence. Oligonucleotides (ONs) targeted to sequences only a few bases apart on the mRNA can give rise to quite different antisense effects. The interaction between target and antisense agent is determined in part by their secondary and tertiary structures, which are difficult to predict[1]. Existing methods for energy calculations do not predict the results that are observed and so there has been a move towards a more empirical approach to antisense site selection. Stull et al. recently reported a study of 37 ONs targeted against murine tumour necrosis factor-α[2]. Each oligonucleotide was individually synthesised and evaluated using a gel-shift binding assay. In another case 100 different 20-mers were synthesized, targeting various genes of HSV-1[3].

We demonstrate a combinatorial technique that allows simultaneous assessment of all possible ONs within a given region identifying sequences open to duplex formation. An oligonucleotide "scanning" array reduces the number of synthesis steps while providing a parallel and exhaustive analysis of all ONs in the region to be targeted. We chose the well-studied example of rabbit β-globin as the target mRNA and synthesised an array of antisense oligodeoxynucleotides against 122 bases at the 5' end. An in vitro transcript was hybridized to the array which comprised all ONs up to a length of 17 bases.

We found that intramolecular base pairing sequesters much of the sequence, preventing the intermolecular pairing essential to antisense activity. However, those ONs which give high duplex yield on the array proved to be effective antisense agents in in vitro RNase H and translation studies.

Results

Hybridization to the scanning array. Under conditions close to equilibrium, hybrid yield for the β-globin mRNA was very low across most of the array (Fig. 1A). There was no detectable hybridization to bases 1 to 37 or 76 to 90 of the mRNA, almost half of the region scanned by the array. Only one sequence gave high duplex yield, a 15-base ON complementary to bases C46–C60. This sequence is also contained within two 16-mer and three 17-mer ONs (Fig. 1B). The yield of these six heteroduplexes was at least three times that of any other ON and five times that of the 17-mer selected by Cazenave et al.[4], located five bases downstream. An area of weak hybridization starts at base C38. These hybrids include bases C40–A44 which are predicted to be unpaired in the RNA (Fig. 2). Two other regions gave weak but detectable hybridization: 14 ONs hybridized to a region starting at base A61 and ending at C91; a second region of weak hybridization starts at base C94. This region contains a run of eight purines, interrupted by a cytosine, with four consecutive guanines which may help to stabilise the heteroduplex.

In a region of 122 bases around the start codon of the β-globin mRNA over 60% of the 106 complementary 17-mer ONs gave duplex yields less than one tenth that of the optimal ON. Sixty-two percent had an intensity less than one tenth that of the highest and all but five had a heteroduplex yield of less than 40% of the highest yield. The top three are derived from the core 15-base optimal ON and the fourth is one base shifted starting at base A48 (Fig. 1B). The fifth ON (in the 40% to 50% range) is the 17-mer starting at base C94: the beginning of an area of moderate hybridization.

In contrast to the result with RNA, single-stranded DNA of the same base sequence and length, which has weaker intramolecular bonding than RNA, hybridized with moderate yield to most of the array (data not shown). This result verified the integrity of the array synthesis.

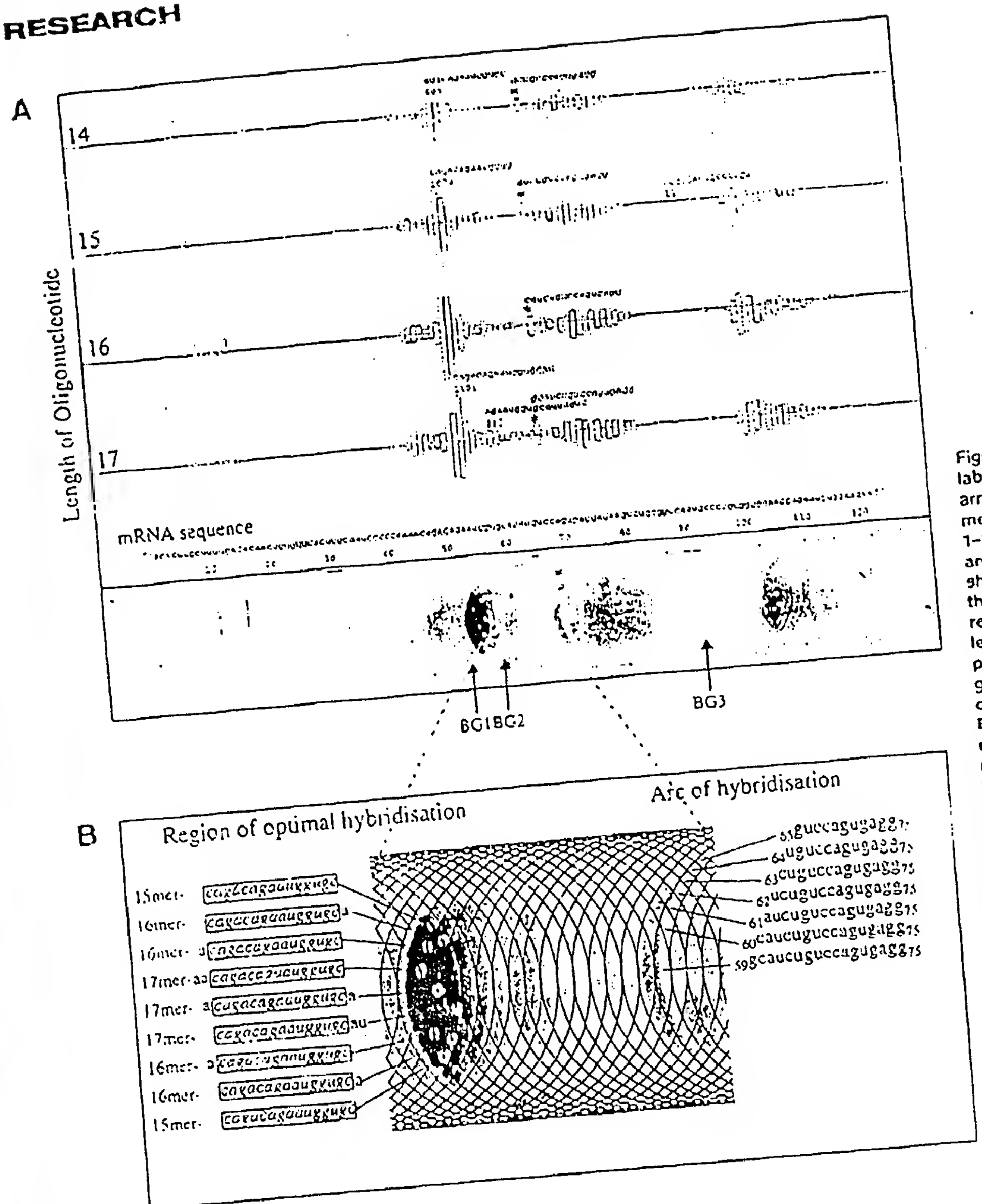

Figure 1. (A) Hybridization of ³²P-labeled β-globin transcript to an array of ONs (monomers to 17-mers) complementary to bases 1–122. The two halves of the array are integrated separately and are shown by bars above and below the center line. Each histogram represents a set of ONs of one length (shown on the left). The positions, sequences and integrated intensity values of the oligonucleotides BG1, BG2, and BG3, used in antisense experiments are indicated. The asterisks mark an arc shaped region of hybridization (see below). The sequences as written represent the mRNA sequence, the complements of the antisense ON sequences. (B) The area of the array around the optimal ON. The core 15-mer sequence is boxed and written in italics. The position and sequence of each ON is indicated. BG1 is the right most 17-mer comprising the 15-mer with an additional "au" at the right hand end. Also shown is the position of the "arc" of oligonucleotides, each of which gives the same yield of heteroduplex and each of which terminates in the same base at the 5′ end.

Antisense activity of oligonucleotides with different binding strengths. Three ONs, with different heteroduplex yields, were chosen for study by in vitro translation and RNase H assay. BG1 was complementary to bases C46–U62 and includes the high yielding 15-mer; a length of 17 bases was chosen for a more direct comparison with Cazenave's ON. BG2 (A51–C67) was the antisense oligonucleotide studied by Cazenave et al. BG3 (C85–U99, gave no detectable hybridization (Fig. 1A).

RNase H mediated cleavage. To compare the effectiveness of BG1 and BG2 at recruiting RNase H, the oligonucleotides were added to labeled β-globin transcript over a range of concentrations (Fig. 3A). At equimolar ratio of ON to mRNA, BG1 caused cleavage of 50% of the transcript as compared to only 9% for BG2. In the higher concentration ranges, similar effects were obtained with tenfold lower concentration of BG1 as compared with BG2. These results indicate that BG1 is five to ten times more effective than BG2.

The rate of reaction could be limited by hybrid formation or by RNase H activity. BG1 and BG2 were added to β-globin transcript at a 100:1 molar excess and sampled over time (Fig. 3B). For BG1 all of the transcript was cleaved by 1 min. For BG2, the reaction

Table 1. The concentrations of ON required to produce 50% inhibition of rabbit β-globin protein product.

Position and designation	Hybrid yield	Oligo concentration	Ratio concentration oligo:β-globin	% Inhib. in WGE
47–62 BG1	980	0.1 μM	4:	50%
51–67 BG2	192	0.5 μM	20:1	50%
85–99 BG3	8	1 μM	40:1	0%
3–19	28	0.2 μM	50:1	50%*
44–54	18	3 μM	750:1	50%**

Concentrations are final concentrations in the translation reaction. The final concentration of β-globin mRNA was 24 nM. In the results taken from references 6 and 16 the concentration was 4 nM.

rate was much slower—at 1 min only 42% of the transcript had been cleaved. The rate difference shows that the reaction rate was limited by heteroduplex formation. Prehybridization of the ON increased the amount of cleavage product over that seen when the ON was added with the enzyme, confirming the dependence of the rate on the hybridization reaction (data not shown).

In vitro translation. Correlation between extent of hybridization and efficacy as antisense reagents extended to in vitro translation in wheat germ extract (WGE) (Fig. 4). At high concentrations (10 µM), each oligonucleotide caused nonspecific inhibition of both α- and β-globin synthesis. However, at 1 µM inhibition was specific: BG1 completely blocked β-globin; BG2 reduced it to 36% and neither affected α-globin translation; BG3 had no effect on either β- or α-globin. Further reduction in concentration showed that for BG1 the minimum concentration needed to give complete, specific inhibition of translation was between 0.1 and 0.2 µM as compared to 1–10 µM for BG2 (data not shown).

Table 1 summarizes the comparison of in vitro translation studies in WGE of ONs targeted to the 5' end of rabbit β-globin. BG1 requires a concentration five times lower than any other ON to effect 50% inhibition of translation.

Discussion

The regulatory cap region is often chosen as an antisense target site. Specifically for β-globin, it is thought to be relatively open and accessible to facilitate high levels of translation[5]. However, we found insignificant level of hybridization to the first 37 bases at the 5' end of the mRNA. Other work (in systems where RNase H is present) has shown that ONs targeted to the cap region of this gene are relatively poor antisense agents[6,7].

The sequence and predicted secondary structure of the mRNA give few clues as to what makes BG1 particularly amenable to duplex formation (Fig. 2). A run of seven ribopurines interrupted by a cytosine, aagacaga, may stabilize the duplex but six bases of this sequence are paired in a relatively stable stem in the predicted RNA structure. Runs of ribopurines are known to stabilize DNA:RNA heteroduplexes[8], but alone are not sufficient to induce heteroduplex formation as bases G72–G80, gaggagaag, four of which are predicted to be unpaired, are present in hybrids which gave only modest yields.

Duplex yield is not necessarily increased by increasing the length of the oligonucleotide; BG1 is a clear example where the 15-mer gives the same yield as cognate 17-mers (Fig. 1B). We have found for other RNAs that short oligonucleotides give higher yield than longer ones. We speculate that the longer oligonucleotides have internal base pairing which prevents heteroduplex formation, or that duplex formation is inhibited by dangling ends of oligonucleotide which cannot fit into the folded structure of the RNA.

It seems likely that duplex formation is confined to those regions in the RNA which provide an accessible substructure. This substructure will include a site for nucleation, which must have unpaired bases. Duplex formation will progress from the nucleation site through a 'zippering' process, and stop when this process meets an energy barrier. Such barriers may include the ends of stems or sharp turns in the folded RNA. The yield of heteroduplex

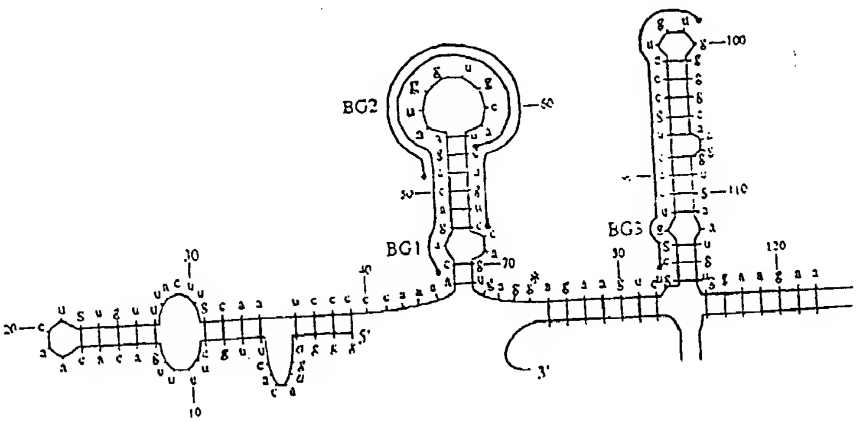

Figure 2. Prediction of the secondary structure of rabbit β-globin mRNA[5]. The region shown is that covered by the scanning array. The 17 RNA polymerase consensus sequence (shown in italics) produces a very small local structural change which should not affect hybridization. The positions of the oligonucleotides used in antisense experiments are indicated. The critical base, G75, in the "arc" pattern of hybridization is marked by an asterisk.

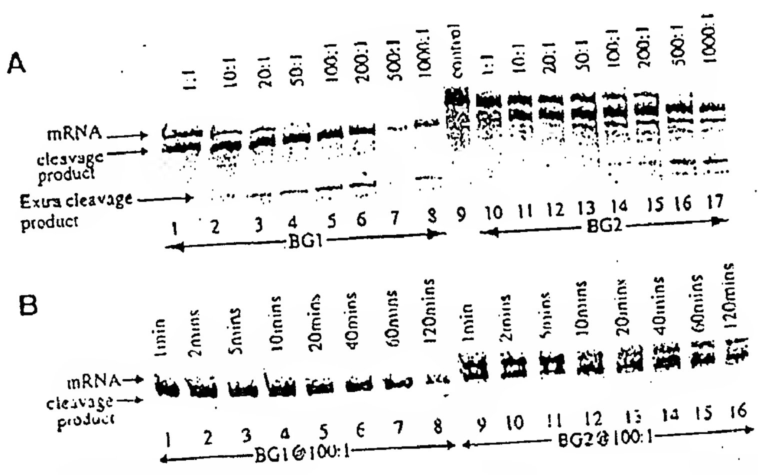

Figure 3. (A) Effect of antisense ON concentration on in vitro RNase H activity against rabbit β-globin mRNA. The full length mRNA of 608 bases and the cleavage product of about 550 bases are indicated. (B) A time-course of in vitro RNase H activity with BG1 and BG2 at 1 µM (a 100:1 molar excess) and transcript at 10 nM. The extra cleavage product is due to a six-base region of complementarity (bases 431–436 of the mRNA) to BG1 and BG2.

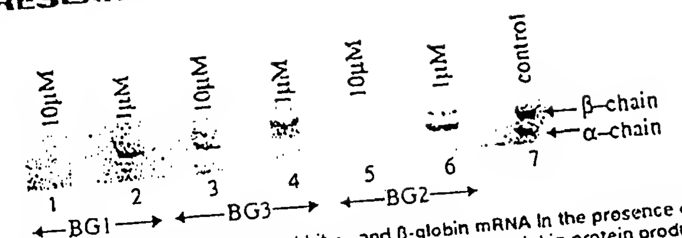

Figure 4. In vitro translation of rabbit α- and β-globin mRNA in the presence of antisense oligos BG1, BG2, and BG3. The upper band is the β-globin protein product and the lower the α-globin protein. The products were analysed by TAU-PAGE[5].

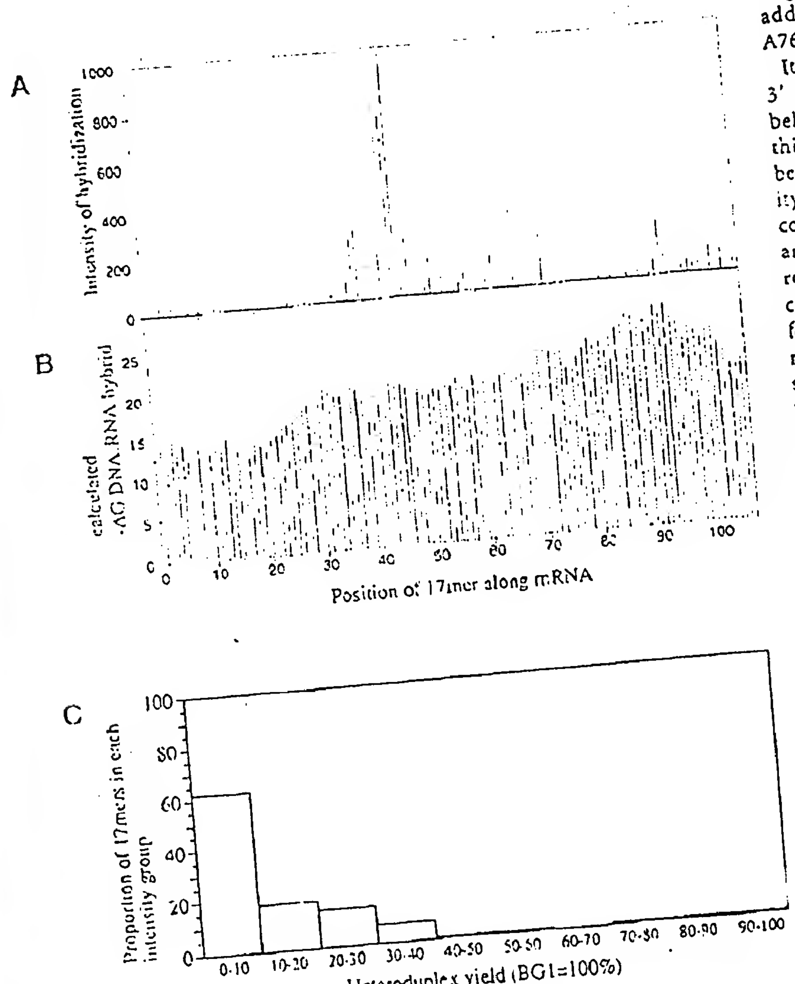

Figure 5. (A) Integrated signal intensities of the 17-mer heteroduplexes across the array, taken from Figure 1A. The first bar corresponds to the heteroduplex yield between the mRNA and the DNA complement of bases A1–C17. The histogram moves sequentially along the mRNA demonstrating the large range in heteroduplex yield. (B) The calculated negative free energies of formation of the 17-mer heteroduplexes. (C) Distribution of heteroduplex yield of the 106 17-mers. The values of the signal intensities were those shown in Figure 1A.

will be determined by the association and dissociation rates: The association rate is determined by the formation of a nucleation complex and subsequent zippering; the dissociation rate is determined by the stability of the formed hybrid. Both of these are affected by structural features in the RNA.

The arc pattern of hybridization reveals an interesting structural element in the RNA (Fig. 1B). The group of ONs in this arc all share the same 5′ end, G75, and reduce in length from the 3′ end. In the structure prediction (Fig. 2) G75 is a free, unpaired base at the end of a stem. With this base at its 5′ end, the ON is apparently able to form a stable duplex by unwinding the two stems upstream of it, U62–C67 and G70–U71. Adding a base to the 5′ end or removing the complement of G75 reduces heteroduplex yield dramatically. G75 may provide a stable nucleation point that is particularly well configured for hybridization to the free 5′ end of the oligonucleotide. Once formed the hybrid may gain added stability by stacking onto the adjacent stem A76–C82.

It is likely that the oligonucleotides tethered at their 3′ ends would nucleate from the free 5′ end, and behave differently from ONs in solution. Although this is a concern, our results gave good correlation between hybrid yield on the array and RNase H activity. In another study, heteroduplex yield on the array correlated well with in vivo and in vitro cell culture antisense activities. Monia et al.[9] tested 34 phosphorothioate ONs mostly in the 5′ and 3′ UTRs for specific inhibition of C-*raf*-1 kinase gene expression, and found one, ISIS 5132, to be an order of magnitude more effective than any other at reducing gene expression in a cell culture assay. ISIS 5132 was also shown to suppress the growth of tumors engrafted in nude mice. In a blind experiment, analysis on a scanning array picked out ISIS 5132 as one of the two high-yielding ONs in 100 base region around ISIS 5132 (data not shown). For the rest of the ONs covered by the array, heteroduplex yield closely followed the antisense activity.

Our results confirm that the susceptibility of a target RNA to antisense interaction varies greatly from one part of the sequence to another and indicate that it is determined by the potential to form heteroduplex. Few sites in the target are open to interaction in the 5′ end of the β-globin mRNA. This is typical of analyses we have done on several mRNAs and in all parts of the sequence (data not shown). At each of the accessible sites very few ONs, often only one, are able to form duplex. These results were not predicted by calculations based on the free energy difference between the reactants—the folded mRNA and the single-stranded ON—and the heteroduplex product. Our calculations were improved over the earlier calculations of Stull et al.[10] as we were able to incorporate recently derived free energy values for RNA:DNA duplexes[11]. The plot for β-globin shows no apparent correlation between the estimated ΔG of heteroduplex formation (Fig. 5B) and the extent of hybrid formed on the array (Fig 5A).

Libraries of random or semi-random ONs have been used to map sites accessible to RNase H along the target mRNA[12,13]. This method reveals open areas, but to pinpoint precise locations Lima et al. individually synthesized many overlapping and different length ONs around the RNase H accessible sites. We envisage a strategy which uses RNase H to scan the whole RNA molecule to locate open regions which can then be examined in detail on oligonucleotide arrays. Wagner et al. recently demonstrated that an

octanucleotide was enough to give specific antisense activity. The exact location was important, suggesting the major determinant to be the RNA structure at the binding site[11]. Taken with our studies these observations make clear the difficulty of finding good candidates for antisense sequences by methods which are not based on experimental measurements.

The method used in this study can be adapted to any analogue for which there is appropriate solid phase chemistry, it has been used to make arrays of phosphorothioate,2'-O-methyl and other derivatives. With other modifications, it could be used for synthesis of peptide nucleic acid analogues (PNAs). It is clear that selecting oligonucleotides which interact well with the target has important consequences for their efficacy as antisense agents. For therapeutic agents this translates into enhanced specificity and a reduction in dose, with attendant reductions in cost and toxicity.

Experimental protocol

Array fabrication. The array was made as described[15]. Briefly, a glass plate (600 mm × 350 mm) was derivatized with a covalently attached hexaethylene glycol linker[16]. Oligonucleotide synthesis was performed directly onto the coated glass using a circular reaction chamber. The diameter of the chamber was 42.5 mm and the offset between base couplings was 2.5 mm, creating oligonucleotides up to a maximum length of seventeen bases. Oligonucleotide synthesis used standard reagents for phosphoramidite chemistry, omitting the capping step. Synthesis was on an adapted Applied Biosystems (Foster City, CA) 381A synthesizer. Deprotection of the array in 30% ammonia solution was carried out as described[15] in a specially constructed nylon chamber.

Hybridization to the array. Hybridization solution (in vitro synthesized and ^{33}P UTP radiolabeled transcript (50 fmol), NaCl (1 M), TE (10 mM, pH 8.0), SDS (0.01%) in a 500 µl volume) was applied evenly along the length of a glass backing plate. The plate carrying the scanning array was placed face down on top of the backing plate ensuring no air bubbles were trapped. The hybridization assembly was placed in a sealed moist chamber, and left to hybridize for 18 h at 30°C. The array was washed briefly in hybridization solution at 30°C, blotted dry and exposed to a storage phosphor screen (Fuji STIII, Tokyo, Japan) for 20 h. The screen was scanned in a Molecular Dynamics (Sunnyvale, CA) 400A PhosphorImager. The analysis and quantitation of the image used xvseq (data not shown). To analyze the resulting image a template comprising a series of overlapping circles is placed over the image (Fig. 1B) and each region within areas defined by the template is integrated. The integrated values represent the intensity of hybridization of each ON and are displayed as histograms. The quantitation was performed using xvseq. The array is symmetrical about the horizontal axis, providing a duplicate measurement for each ON. The array was stripped of hybridized target by soaking in TE/SDS at 55°C to 65°C, ready for reuse.

Oligonucleotide preparation. Oligodeoxyribonucleotides were synthesised using an Applied Biosystems 381A synthesizer with trityl on and purified using ABI oligonucleotide purification columns (OPC). Purity was evaluated by analysis on a 20% denaturing polyacrylamide gel (1xTBE, 1500V, 30mA, 1.5 h) after 5' terminal labelling with T4 polynucleotide kinase and γ^{33}P-ATP. The sequences of oligonucleotides used were: BG1 5'-atgcaccattctgtctg-3' BG2 5'-gacagatgcaccattct-3' BG3 5'-acagggcagtgaccg-3'.

Preparation of labeled RNA transcript. A double stranded DNA template carrying the T7 transcription promoter sequence was generated using the RT-PCR protocol of Maniatis et al.[17] using purified rabbit globin mRNA (Gibco BRL, Paisley, UK). The PCR product was purified using a Pharmacia (Uppsala, Sweden) Microspin column, and transcribed using the standard transcription protocol incorporating 20 µCi α ^{33}P-UTP (1000–3000 Ci/mmol). The product was purified by G25 Sephadex column chromatography. The molecular weight of the transcript was evaluated prior to hybridization by electrophoresis on a 6% denaturing polyacrylamide gel (1xTBE, 1500 V, 30 mA, 1.5 h).

Ribonuclease H reactions. Reactions were according to manufacturer's instructions using 10 nM labeled in vitro transcript and various concentrations of oligonucleotides. The reagents were incubated at 30°C and the reaction was started by addition of the oligonucleotide. Reactions were for 2 h and stopped by the addition of formamide (30%), EDTA (0.2 M). For time-course assays, a 40 µl reaction volume was used. The products were analyzed on a 6% denaturing polyacrylamide gel. Quantitation used Molecular Dynamics ImageQuant software.

In vitro translation. Rabbit globin mRNA (0.5 µg) was added to a translation mixture containing wheat germ extract, amino acids (80 µM each), potassium acetate (100 mM) and oligonucleotides at various concentrations. The reaction was started by the addition of 15 µCi ^{35}S methionine (1000 Ci/mmol) and incubated for 2 h at 25°C. The final concentration of β-globin mRNA in the reaction was 24 nM. Products were analysed by gel electrophoresis on a 12% acrylamide gel containing 0.7% Triton-X100 and 6 M urea, in 5% acetic acid buffer using the method described by Rovera et al.[18]

Acknowledgments

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APPENDIX B

Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-*raf* kinase

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Substantial evidence exists supporting a direct role for *raf* kinases in the development and maintenance of certain human malignancies. Here we test the potential of phosphorothioate antisense oligodeoxynucleotides targeted against human C-*raf*-1 kinase to specifically inhibit C-*raf*-1 kinase gene expression and tumor progression in cell culture and *in vivo*, using human tumor xenograft mouse models. Treatment of human tumor cells with appropriate phosphorothioate antisense oligodeoxynucleotides led to specific inhibition of C-*raf* kinase gene expression in cell culture and *in vivo* at well-tolerated doses. Moreover, oligodeoxynucleotide treatment resulted in potent antiproliferative effects in cell culture and potent antitumor effects *in vivo* against a variety of tumor types that were highly consistent with an antisense mechanism of action for these compounds. These studies strongly suggest that antisense inhibitors targeted against C-*raf*-1 kinase may be of considerable value as antineoplastic agents that display activity against a wide spectrum of tumor types at well-tolerated doses.

Raf genes code for serine threonine-specific protein kinases that play pivotal regulatory roles in proliferative signaling events within the mitogen-activated protein (MAP) kinase signaling cascade[1-4]. Substantial evidence supports a direct role for *raf* kinases in the development and maintenance of certain human malignancies. First, the MAP kinase signaling cascade has been shown to be essential for cellular proliferation and mediation of cellular transformation by most oncogenes[1,2]. Second, *raf* proteins have been shown to be direct downstream effectors of *ras* protein function within the MAP kinase signaling pathway[3-6]. Because *ras* mutations are present in a high proportion of human cancers[7,8], novel therapies directed against *raf* kinases may prove useful in the treatment of *ras*-dependent tumors. Furthermore, mutations in *raf* genes have been shown to transform cells *in vitro*[9-12] and have been associated with certain human tumors[13-15]. Finally, expression of unusually high levels of C-*raf* kinase messenger RNA and protein have been reported in certain human tumors[15-17].

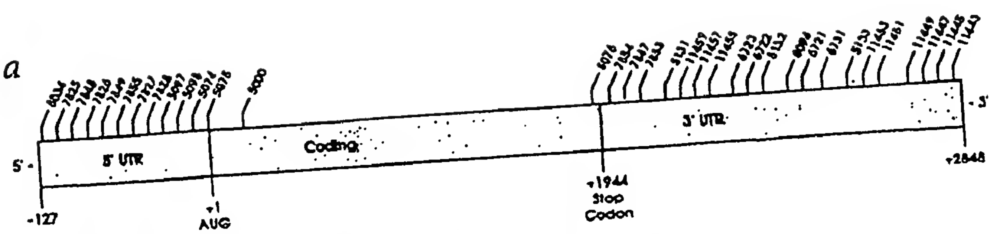
Although significant progress has been achieved in the identification of gene products implicated as causal factors in the onset or maintenance of human tumorigenesis, the emergence of novel therapies that specifically reverse the oncogenic effects of these gene products has generally been slow. Despite the fact that a number of potential limitations for antisense technology have recently been reported[18,19], this rational approach to novel drug discovery remains very attractive for the treatment of a variety of human diseases including cancer[20]. Antisense oligonucleotides, which act by specifically hybridizing with complementary RNA sequences, inhibit protein expression through a number of mechanisms including degradation of the targeted RNA through RNase H-mediated cleavage[20,21]. These properties suggest that antisense oligonucleotides may represent a novel class of antineoplastic agents which, when targeted to appropriate molecular targets, can prevent the initiation or progression of specific human cancers.

In the present study, we test the potential of phosphorothioate antisense oligodeoxynucleotides (ODNs) targeted against human C-*raf*-1 kinase (C-*raf*) to specifically inhibit C-*raf* gene expression and tumor progression in cell culture and *in vivo* using tumor xenograft models. We demonstrate that treatment of human tumor cells with appropriate phosphorothioate antisense ODNs leads to specific inhibition of C-*raf* gene expression in cell culture and *in vivo*. Moreover, antisense-mediated inhibition of C-*raf* gene expression in human tumor cells results in potent antiproliferative effects in cell culture and potent antitumor activity *in vivo*.

Antisense-mediated inhibition of C-*raf* mRNA expression

To identify effective antisense ODNs capable of inhibiting C-*raf* gene expression in human tumor cells, 34 phosphorothioate ODNs, each 20 bases in length and predicted to hybridize with human C-*raf* mRNA, were synthesized. These ODNs were designed to hybridize with various regions of C-*raf* mRNA, with 11 ODNs targeted to the 5'-untranslated region, 3 targeted to sites within the protein coding region, and 20 targeted to the 3'-untranslated region (Fig. 1). Because phosphorothioate ODNs have been shown to commonly act through an RNase H-dependent mRNA cleavage mechanism in cells[20,21], antisense activity for each of these ODNs was determined initially by northern blot analysis probing for levels of C-*raf* mRNA.

The levels of C-*raf* mRNA expression in A549 lung carcinoma cells treated with the antisense ODNs at a concentration of 200 nM in the presence of cationic lipid (DOTMA:DOPE) are shown in Fig. 1. Cationic lipid formulation was used in cell culture



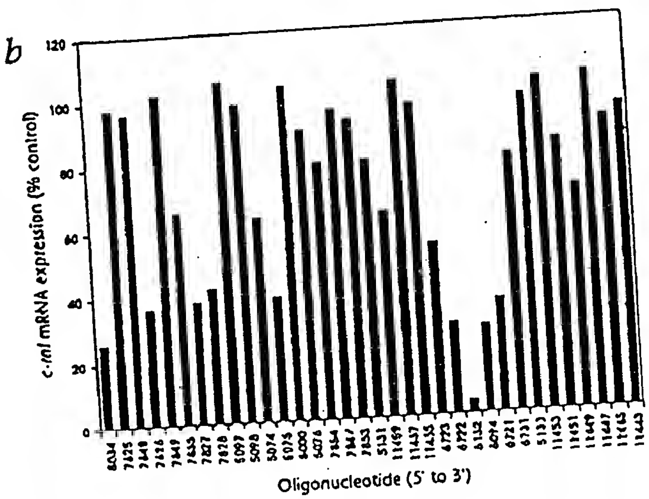
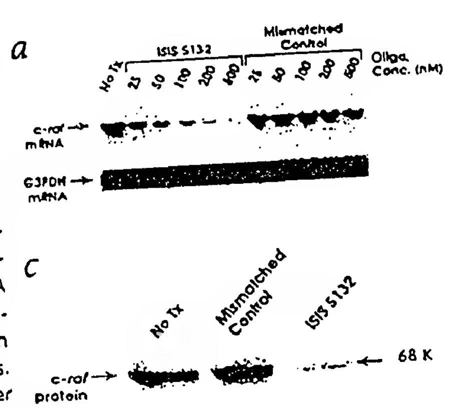


Fig. 1 Reduction in C-*raf* kinase mRNA expression in A549 lung carcinoma cells following treatment with appropriate phosphorothioate antisense ODNs. A549 cells grown in culture were treated with the indicated antisense ODN (200 nM) and C-*raf* mRNA levels were determined 24 h later by northern blot analysis. *a*, Relative positioning of the predicted hybridization sites within the human C-*raf* mRNA of 34 antisense ODNs that were evaluated for their ability to inhibit C-*raf* mRNA expression in cultured tumor cells. *b*, C-*raf* mRNA levels in A549 cells following treatment with phosphorothioate antisense ODNs targeted to C-*raf* mRNA. Cells were treated with the various ODNs at a concentration of 200 nM and RNA was prepared for northern blot analysis 24 h later. C-*raf* mRNA levels are expressed as a percentage of the levels of C-*raf* mRNA untreated control cells. C-*raf* mRNA levels were analyzed, quantified and normalized to G3PDH mRNA levels as described in the Methods section. ODN sequences will be made available from the authors upon request.

experiments to facilitate intracellular uptake of ODNs (ref. 22). Reduction in C-*raf* mRNA levels was observed following treatment with certain antisense compounds targeted to either the 5'-untranslated region, the coding region or the 3'-untranslated region. However, the degree of activity exhibited by different ODNs varied greatly. Most ODNs had little or no effect on C-*raf* mRNA levels, whereas others exhibited moderate effects. The most potent antisense inhibitor identified from this series of ODNs was ISIS 5132, which targets the 3'-untranslated region of C-*raf* mRNA. ODNs targeted to regions of the C-*raf* mRNA immediately flanking the ISIS 5132 hybridization site also inhibited C-*raf* mRNA levels, suggesting that this region of the C-*raf* message is particularly sensitive to antisense-mediated effects. All of the ODNs described here were also tested against other human tumor cell lines (T24 bladder carcinoma, SW480 colon carcinoma) for effects on C-*raf* mRNA levels with essentially identical results. In all cases, ISIS 5132 was determined to be the most potent ODN for reducing C-*raf* mRNA levels in cultured human cell lines. In subsequent studies, the effects of treating cells with shortened ODN analogues of ISIS 5132 (15–19 bases) on C-*raf* expression were also examined. These short analogues were all found to be significantly less effective in reducing C-*raf* expression relative to ISIS 5132. Similar observations have been made using phosphorothioate ODNs targeted against the Ha-*ras* oncogene[22].

Specificity of C-*raf* antisense inhibition

To examine the specificity of ISIS 5132-mediated effects on C-*raf* gene expression, the effects of ISIS 5132 and a mismatched con-



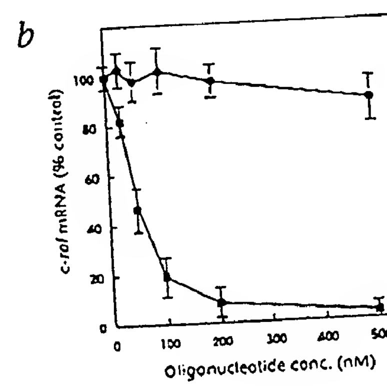


Fig. 2 Inhibition of C-*raf* kinase mRNA and protein expression by ISIS 5132 in A549 cells. *a*, A549 cells were treated with increasing concentrations (25–500 nM) of ISIS 5132 (TCCCGCCTGTGACATGCATT) or a mismatched control analogue of ISIS 5132 that contains seven base changes (mismatches) within the 5132 sequence (TCCCGCGCACTTGATGCATT), and total RNA was prepared 24 h later and anaylzed for C-*raf* and G3PDH mRNA levels by northern blot analysis. No Tx indicates untreated cells. *b*, Quantification of C-*raf* mRNA levels after normalization to G3PDH mRNA levels in A549 cells following treatment with increasing concentrations of ISIS 5132 (■) or a mismatched control analogue of ISIS 5132 (●). Quantification was performed as described in the Methods section. Each point represents the mean of triplicate analyses with error bars as indicated. *c*, Western blot analysis of C-*raf* kinase protein levels in A549 cells treated with ISIS 5132 or the control phosphorothioate ODN. Cells were treated with ODN at a concentration of 200 nM, protein extracts were prepared 48 h later, and C-*raf* protein levels were determined as described in the Methods section.

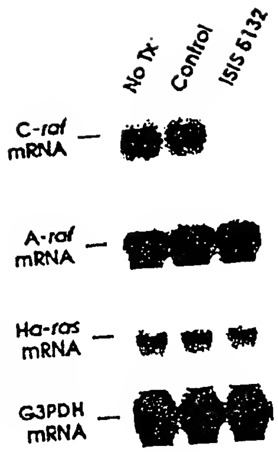


Fig. 3 Target-specific inhibition of C-*raf* mRNA expression in cells treated with ISIS 5132. Northern blot analysis of C-*raf* kinase, A-*raf* kinase, Ha-*ras* and G3PDH mRNA levels in A549 lung carcinoma cells treated with ISIS 5132 or a mismatched control phosphorothioate ODN analogue of ISIS 5132 (control). ODNs were administered at a concentration of 200 nM, and mRNA was prepared and analyzed 24 h later, as described in the Methods section. The sequence of the control ODN is described in the legend to Fig. 2.

trol ODN on C-*raf* mRNA and protein levels were determined (Fig. 2). Treatment of A549 cells with ISIS 5132 caused a dose-dependent reduction in C-*raf* mRNA levels, displaying a median inhibitory concentration IC_{50} for this effect of approximately 50 nM. No effects on C-*raf* mRNA expression were observed in cells treated with the mismatched control analogue at any of the employed ODN concentrations, even when tested at a concentration 10 times that of the IC_{50} for C-*raf* mRNA reduction by ISIS 5132. Studies have also been performed examining the effects of many additional mismatched ODN analogues of ISIS 5132 on C-*raf* gene expression with essentially identical results (unpublished observations).

Inhibition of C-*raf* protein levels (>80%) was also observed in A549 cells treated with ISIS 5132 (Fig. 2*c*). As seen for the effects on C-*raf* mRNA expression, reduction in C-*raf* protein expression by ISIS 5132 was concentration dependent (IC_{50} = 100 nM), and no effects on C-*raf* protein levels were observed in cells treated with a mismatched phosphorothioate ODN analogue of ISIS 5132. However, in contrast to the effects of ISIS 5132 on C-*raf* mRNA expression, maximum reduction in steady-state C-*raf* protein levels required a relatively long post-ODN treatment period (40 hours or greater) and a second application of ODN 24 hours following the initial treatment to maintain reduced C-*raf* mRNA levels. These observations are most likely explained by a relatively long protein half-life for C-*raf* in cells coupled with the fact that phosphorothioate ODNs have a finite half-life in cells due to their susceptibility to nucleolytic degradation[14,15]. Similar effects on C-*raf* protein expression were also observed in other human cell lines treated with ISIS 5132.

To further examine the specificity of inhibition of antisense ODNs targeted to C-*raf* mRNA, the effects of ISIS 5132 on expression of other cellular mRNAs was determined. The effects of ISIS 5132 and a mismatched control ODN on the expression of C-*raf* mRNA, A-*raf* kinase mRNA, Ha-*ras* mRNA and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA in A549 cells treated with ODN at a concentration of 200 nM are shown in Fig 3. Because no sequence similarities exist between the ISIS 5132 target sequence and sequences within human A-*raf*, Ha-*ras* and G3PDH mRNA, ISIS 5132 would not be expected to affect expression of these cellular mRNAs if it was acting through an antisense mechanism. ISIS 5132 was found to completely eliminate expression of C-*raf* mRNA in a highly sequence-specific and target-specific manner. No effects were observed on expression of the other examined cellular mRNAs in cells treated with ISIS 5132 or its mismatched control ODN.

Antiproliferative effects of C-*raf* antisense inhibition

To determine the effects of ISIS 5132 on cell proliferation, we treated A549 cells one time with either ISIS 5132 or the mismatched control ODN at a concentration of 200 nM and determined cell number daily over a 5-day period. Treatment of cells with ISIS 5132 resulted in marked inhibition of A549 cell proliferation over this 5-day period (Fig. 4*a*). Cell growth was not influenced significantly by the treatment of cells with the mismatched control ODN. Interestingly, the rate of cell proliferation began to approach control values 4 and 5 days after treatment with ISIS 5132. This recovery of proliferation rate at days 4 and 5 after ISIS 5132 treatment correlated well with the recovery of C-*raf* mRNA expression due to nuclease-mediated degradation of ISIS 5132 following a single application of the ODN.

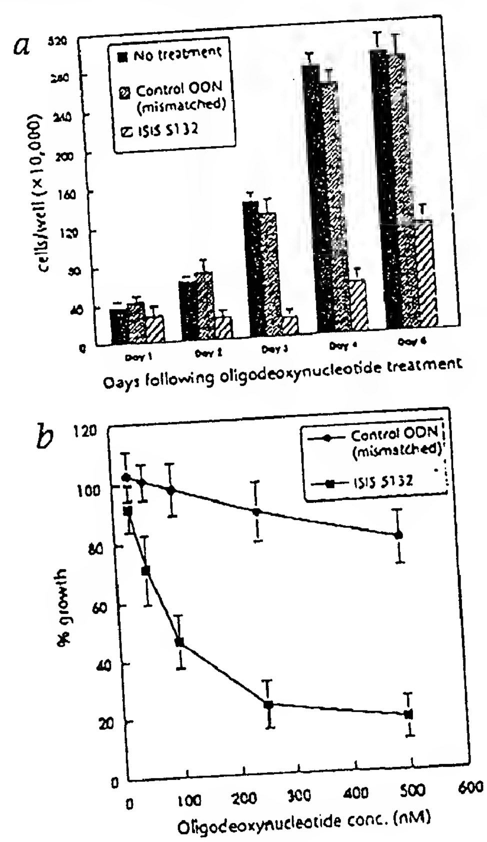


Fig. 4 Antiproliferative effects of C-*raf* antisense inhibition by ISIS 5132 in A549 cells. *a*, Time course for the antiproliferative effects of ISIS 5132 in A549 cells. Cells were treated one time at time zero with 200 nM ISIS 5132 or a mismatched control ODN analogue of ISIS 5132 (described in Fig. 2) and cell number was determined at days 1, 2, 3, 4 or 5 following ODN treatment (as described in the Methods section). *b*, Dose-response curve for A549 growth inhibition by ISIS 5132 (L). Cells were treated with increasing concentrations of ODNs at time zero, and cell number was determined 3 days later as described in the Methods section. Percent inhibition was calculated by comparison with the number of cells present in cultures grown in the absence of ODNs. Mismatched control (P) is the same as that described in Fig. 2. For both *a* and *b*, each point represents the mean of triplicate cultures with error bars as indicated.

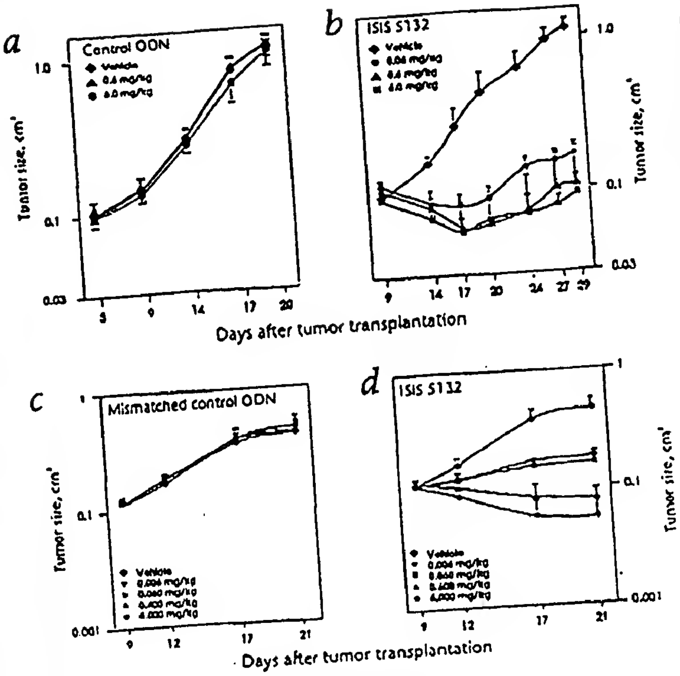


Fig. 5 Effects of ISIS 5132 administration on the growth of A549 tumors in nude mice. A549 tumors were established subcutaneously in nude mice over a 5- to 9-day period, as described in the Methods section. Following establishment of tumors, ODNs prepared in saline solution were administered once daily by bolus intravenous injection at the indicated doses and tumor size was determined and tumor volume was calculated over a 3-week period following initiation of ODN treatment. *a*, Effects of a control, 21-base, phosphorothioate ODN targeted to a herpes simplex virus gene product (GCGGAGGTCCATGTCGTACGC) on the growth of A549 tumors in nude mice. ODN administration was initiated on day 5 and continued for 13 days. *b*, Effects of ISIS 5132 on the growth of A549 tumors in nude mice. ODN administration was initiated on day 9 and continued for 19 days thereafter. *c*, Effects of a mismatched ODN analogue of ISIS 5132 (sequence described in Fig. 2) on the growth of A549 tumors in nude mice. *d*, Effects of ISIS 5132 when tested over a larger dose range on the growth of A549 tumors in nude mice. For panels *c* and *d*, ODN administration was initiated on day 9 and continued for 11 days thereafter. Each point represents the mean tumor volume in experimental groups containing at least six animals per group. The results are representative of eight independent experiments.

The effects of ISIS 5132 on cell proliferation were also found to be dose dependent over a concentration range between 25 nM and 500 nM (Fig. 4*b*). The IC_{50} for these antiproliferative effects was between 50 nM and 100 nM. This IC_{50} for inhibiting A549 proliferation correlated well with the IC_{50} required to inhibit C-*raf* mRNA expression by ISIS 5132 (Fig. 2), strongly suggesting that the antiproliferative effects observed for ISIS 5132 are a direct result of the inhibitory effects of this ODN on C-*raf* gene expression. No significant effects were observed on proliferation in cells treated with the mismatched control ODN at any of the tested concentrations.

Antitumor activity of ISIS 5132 *in vivo*

The effects of ISIS 5132 on human tumor growth *in vivo* was next examined using subcutaneously implanted tumor xenografts in nude mice. In these studies, ODNs, prepared in saline solution, were administered to tumor-bearing mice by bolus intravenous injection once daily. ODN administration was initiated between 5 and 9 days following tumor fragment implantation, at which time tumors were approximately 100^3 mm in size. The effects of ISIS 5132 administration on the growth of A549 tumors in mice over a range of doses are shown (Fig. 5). In initial experiments, ISIS 5132 was tested at doses ranging between 0.06 mg per kg body weight and 6.0 mg/kg and compared with the effects of a control phosphorothioate ODN 21 bases in length targeted to a herpes virus gene product[26]. ISIS 5132 displayed very potent inhibitory effects on the growth of A549 tumors in mice, whereas no effects on tumor growth were observed for the control ODN (Fig. 5, *a* and *b*). To further characterize the dosing requirements and the specificity of ISIS 5132-mediated antitumor activity, the dosage range of ODN administration was expanded to include a dose one-tenth that of the previous experiment (0.006 mg/kg), and a mismatched control ODN analogue of ISIS 5132 was included. The effects of ISIS 5132 on tumor growth was highly

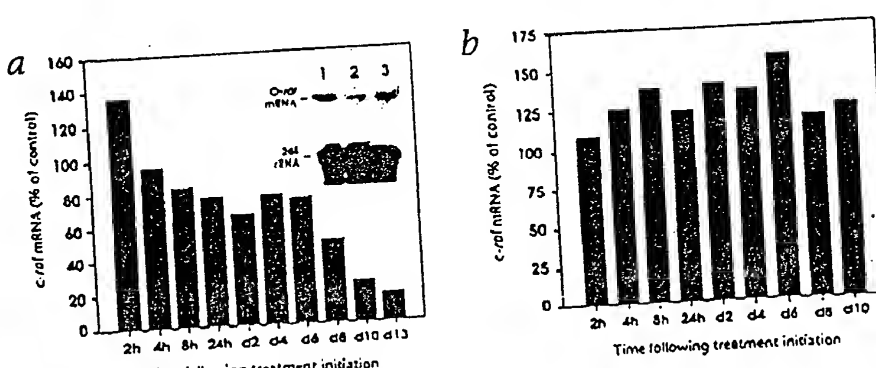


Fig. 6 Effects of ISIS 5132 administration on C-*raf* kinase mRNA levels in A549 tumors in nude mice. Total RNA was prepared from subcutaneously growing A549 tumors in nude mice at various periods of time following initiation of ODN treatment and C-*raf* mRNA levels were determined by northern blot analysis. C-*raf* mRNA levels were quantified by PhosphorImage analysis and normalized to G3PDH mRNA levels, as described in the Methods section. *a*, Effects of ISIS 5132, administered once daily (beginning at time zero) at a dose of 6.0 mg/kg, on C-*raf* mRNA levels in A549 tumors in nude mice. *b*, Effects of a mismatched ODN analogue of ISIS 5132 (sequence described in Fig. 2), administered once daily (beginning at time zero) at a dose of 6.0 mg/kg, on C-*raf* mRNA levels in A549 tumors. *Inset in a*, C-*raf* mRNA levels in A549 tumors at day 10 following administration of ISIS 5132 or the mismatched ODN at a daily dose of 6.0 mg/kg in nude mice. Lane 1, no treatment; lane 2, ISIS 5132 treatment; lane 3, treatment with the mismatched ODN. The results are representative of two independent experiments.

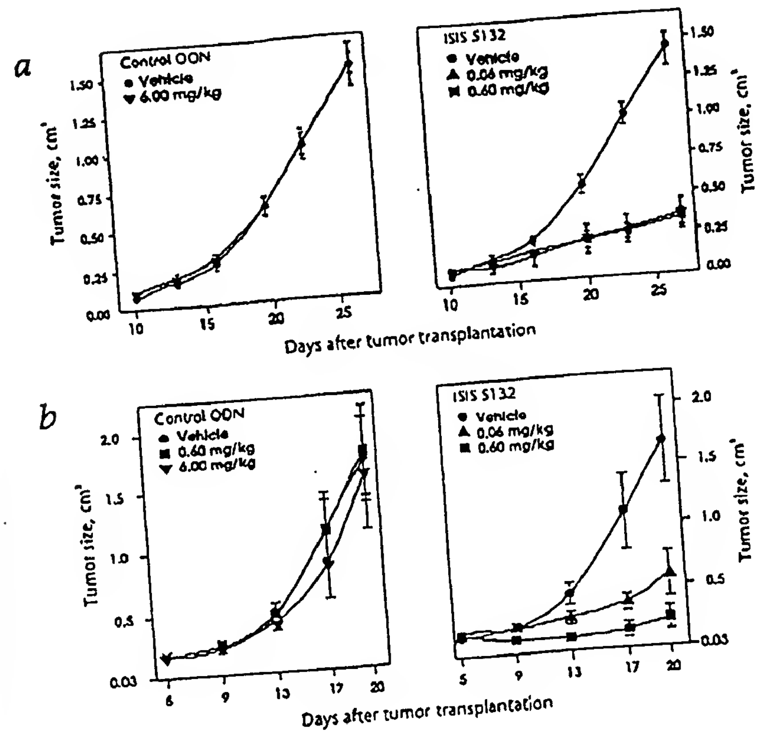


Fig. 7 Effects of ISIS 5132 administration on the growth of T24 and MDA-MB-231 tumors in nude mice. Tumors were established subcutaneously in nude mice over a 5- to 10-day period, as described in the Methods section. Following establishment of tumors, ODNs prepared in saline solution were administered once daily by bolus intravenous injection at the indicated doses and tumor size was determined and tumor volume calculated over a 3-week period following initiation of ODN treatment. *a*, Effects of ISIS 5132 or a control phosphorothioate ODN targeted to a herpes simplex virus gene product (sequence described in Fig. 5) on the growth of subcutaneously implanted MDA-MB-231 (breast) tumors in nude mice. ODN administration was initiated on day 10 and continued for 14 days thereafter. *b*, Effects of ISIS 5132 or the herpes virus control ODN on the growth of subcutaneously implanted T24 (bladder) tumors in nude mice. ODN administration was initiated on day 5 and continued for 14 days thereafter. Each point represents the mean tumor volume in experimental groups containing at least six animals per group. The results are representative of three independent experiments.

dose-dependent, displaying potent antitumor activity at a dose as low as 6.0 µg/kg (Fig. 5*d*). The IC_{50} value for antitumor activity of ISIS 5132 against the A549 tumor xenografts ranged between 0.06 and 0.6 mg/kg. Moreover, no antitumor effects were observed following treatment with identical doses of the mismatched control phosphorothioate ODN (Fig. 5*c*).

The antitumor effects described above for ISIS 5132 along with the lack of antitumor effects observed for the various control ODNs supports the conclusion that the antitumor effects observed for ISIS 5132 occur through an antisense mechanism of action. To further test this conclusion, we examined the effects of ISIS 5132 on C-*raf* mRNA expression in A549 tumors over time in mice treated with ISIS 5132 or the mismatched control ODN. In this experiment, tumor-bearing mice were administered ODN by bolus intravenous injection once daily at a dose of 6.0 mg/kg and total RNA was prepared from tumors over time, ranging between 2 hours and 13 days following initiation of ODN treatment, and C-*raf* mRNA levels were determined by northern blot analysis. Administration of ISIS 5132 to mice resulted in a time-dependent reduction in C-*raf* mRNA levels in tumors (Fig. 6*a*). Reduced C-*raf* mRNA levels were observed within hours following administration of ISIS 5132, but maximal inhibition of mRNA expression required continued treatment with maximal effects requiring 13 days of ODN administration. Moreover, no effects were observed on C-*raf* mRNA levels in mice treated with the mismatched control ODN (Fig. 6*b*).

The *in vivo* antitumor effects of ISIS 5132 against two additional human tumor cell lines were also examined: the MDA-MB-231 breast carcinoma cell line and the T24 bladder carcinoma cell line[27,28]. Administration of ISIS 5132 to tumor-bearing mice dramatically inhibited the growth of MDA-MB-231 and T24 tumors at doses ranging between 0.06 mg/kg and 6.0 mg/kg (Fig. 7). As seen for the effects observed in A549 tumor xenografts, no antitumor effects were observed in animals during treatment with the control phosphorothioate ODN.

Discussion

We have shown that administration of properly designed phosphorothioate antisense ODN inhibitors against C-*raf* markedly inhibits expression of C-*raf* in cell culture and *in vivo*. The most potent antisense inhibitor against C-*raf* identified in this study was ISIS 5132, a 20-base phosphorothioate ODN targeted to the 3'-untranslated region of human C-*raf* mRNA. This ODN, which was used as the C-*raf* antisense inhibitor for all subsequent studies, displayed an IC_{50} value between 50 nM and 100 nM for inhibiting C-*raf* mRNA expression and tumor cell proliferation in cell culture and an IC_{50} value between 0.06 mg/kg and 0.6 mg/kg for inhibiting tumor growth *in vivo* against three different tumor types when administered once daily by intravenous injection. Activity of antisense ODNs in cell culture required the use of cationic lipids to facilitate intracellular delivery of ODNs, whereas lipid formulations were not required for the effects of ISIS 5132 on C-*raf* gene expression and tumor growth *in vivo*. This observation is consistent with previous reports demonstrating that, although ODN transfection methods are required *in vitro*, these procedures are not required to elicit antisense ODN effects *in vivo*[22,29,30]. Similar observations have been reported for the uptake and expression of plasmid DNA in mice[31]. Administration of ISIS 5132 to tumor-bearing mice was also shown to inhibit C-*raf* mRNA expression in tumors, whereas control phosphorothioate ODNs had no such effects. In other studies, we have examined the presence and integrity of ISIS 5132 and control phosphorothioate ODNs in various tissues when administered at effective doses to tumor-bearing mice and have observed significant, sequence-independent accumulation of intact ODNs, along with their expected metabolites[32,33], in tumors and in various host tissues. In these studies we have observed accumulation of ISIS 5132 at 10–50 µM concentrations in tumors (A549 and MDA-MB-231) in which animals received a single 6.0 mg/kg dose of ISIS 5132 by intravenous injection. As the ISIS 5132 hybridization site

within human C-*raf* mRNA is not conserved in the murine species (personal communication, D. Morrison, and unpublished experiments), no effects were observed on C-*raf* mRNA expression in host tissue.

One of the most attractive features of antisense-based inhibitors is the potential for great specificity in their inhibitory effects against the intended molecular target[20]. Demonstration of such specificity is usually considered to be the most important criterion for concluding true antisense mechanisms underlying the biological effects of a particular ODN. For this reason, we have examined in considerable depth the specificity of inhibition of C-*raf* gene expression and tumor growth by ISIS 5132 and have demonstrated that the effects of this ODN are highly specific as measured by a number of parameters. First, during a screen of more than 30 phosphorothioate ODNs complementary to various sites within the C-*raf* mRNA, only a few were found to exhibit potent inhibitory effects on C-*raf* expression. Because RNA strucrure has been shown to have profound effects on ODN hybridization efficiency[33-35], as well as the efficacy of antisense ODNs[36,37], these results are most likely due to the presence of a limited number of optimal ODN hybridization sites within the structured C-*raf* mRNA. Second, a mismatched phosphorothioate analogue of ISIS 5132 had no effects on C-*raf* mRNA expression in cell culture or *in vivo*, and had no effects on tumor growth in cell culture or *in vivo*. We have also determined the effects of other mismatched control phosphorothioate analogues of ISIS 5132 for inhibition of C-*raf* gene expression in cell culture and antitumor activity *in vivo* and have observed effects that are consistent with rhe effects of the ISIS 5132 mismatched ODN described in this report (unpublished observations). In addition to ODN sequence specificity, we have demonstrated that the effects of ISIS 5132 on C-*raf* mRNA expression were specific for the intended target in thar no effects were observed on the expression of any other cellular mRNA examined, including a related isotype, A-*raf* kinase. Finally, we have addressed recent suggestions thar the anritumor activity conferred by certain anrisense ODNs may be due in part to a stimulatory effect on natural killer (NK) cell activity in mice[38-40] by demonstrating that ISIS 5132 is completely devoid of NK cell stimulatory activity (R. Boggs *et al.*, manuscript in preparation). These findings, along with the many other observations enumerated in this report, strongly suggest that the inhibitory effects of ISIS 5132 on C-*raf* expression and the biological consequences that appear to result from these inhibitory effects in cell culture and *in vivo* occur through an antisense mechanism of action. Nevertheless, phosphorothioate ODNs have been shown under certain conditions ro exert effects through mechanisms other than antisense[43-45] and, therefore, additional mechanisms underlying the actions of ISIS 5132 cannot be ruled out at the present time.

One of the potenrial limitations of a pharmacological agent targeted against an importanr cellular regulatory protein is the potential roxiciry that will resulr from the drug's action in nondiseased tissues ("exaggerated pharmacology"). We have addressed this issue directly for C-*raf* antisense inhibitors by identifying potent murine-specific C-*raf* antisense compounds and testing them in mice under subacute and chronic treatment conditions. Although we have been able to demonstrate significantly reduced levels of C-*raf* mRNA and protein in mouse tissues following administration of these compounds, we have been unable to demonstrate significanr toxicities resulting from these effects (B.P.M. & J.F.J., manuscript in preparation). These results suggest that tumors may be more sensitive to C-*raf* inhibition relative to normal tissues and that pharmacological agents specifically designed to inhibit C-*raf* expression in cells may display a relatively large therapeutic index for the treatment of cancer in the clinic.

The ability to use antisense ODNs to target selectively the genetic processes involved in cancer has raised the exciting possibility that these compounds could be used, not only as a new class of chemotherapeutic agent to combat cancer, but also to gain a better understanding of the critical molecular events responsible for initiating or maintaining the cancer phenotype. *Raf* kinases have been implicated as critical mediators of oncogenic transformation due to their central regulatory role in the MAP kinase signaling pathway[1,2]. The studies reported here support this conclusion. Furthermore, because the inhibitory effects of ISIS 5132 on *raf* gene expression are specific and limited to the C-*raf* isotype, our findings suggest that inhibition of this isotype is sufficient to elicit potent antitumor effects against the three tumor types examined in this study. The recent discovery that *raf* kinases function in part as downstream mediators of *ras* oncogene action[3-6] suggests that inhibitors of *raf* gene expression may prove useful in the treatment of *ras*-dependent tumors. Of the three tumor types shown here to be responsive to the antitumor effects of ISIS 5132 (A549 lung, T24 bladder, MDA-MB-231 breast), two (T24 and A549) have been reported to conrain *ras* mutations[41,42], supporting the premise that C-*raf* inhibition may be useful for the treatment of human tumors thar are associated with these mutations. However, as the incidence of *ras* mutations in human breast carcinomas has been shown to be a relatively rare event[7,8], and as ISIS 5132 was found to be highly effective against the breast rumor line MDA-MB-231, it is possible that the antitumor effects resulting from C-*raf* inhibition will not be limited to *ras*-dependent tumors but will include a much broader spectrum of tumor types. Consistent with this possibility are our findings that antisense ODNs targeted againsr *ras* gene products display potent antitumor activity againsr A549 and T24 tumor xenografts at a level that is nearly equal to that of the antitumor activity displayed by ISIS 5132, but show relatively poor activity against MDA-MB-231 tumor xenografts.

Despite the posirive outlook for antisense inhibitors targeted against C-*raf* for the treatment of cancer, some potential limitations may exist for rhis approach. For example, little is known about the potential for developmenr of resisrant tumor cell populations that lose their sensitivity toward C-*raf* inhibition over time. One possible mechanism by which such resistance may be acquired is through the recruitment of alternate signaling pathways, possibly involving the activity of other *raf* kinase isotypes. Because virtually all drugs encounter some degree of resistance in target cell populations, resistance to antisense drugs is likely to be encountered to some extent in the clinic. In addition, some tumor cells may never display sensitivity toward C-*raf* inhibition. For example, several reports exist documenting the presence of *raf* kinase-independent *ras* signaling pathways that are essential for a variety of cellular functions[46-48]. Tumor cells that are solely dependent on such pathways will likely be unaffected by inhibitors against C-*raf*. Thus, studies examining the effects of ISIS 5132 against a broader spectrum of tumor types and characterization of the antitumor properties of antisense inhibitors designed against other *raf* kinase family members and other members of the MAP kinase signaling pathway, both alone and in combination, are of obvious importance.

Methods

Oligonucleotide synthesis. All oligonucleotides used in these studies were phosphorothioate oligodeoxynucleotides (ODNs) and their sequences will be made available from the authors upon request. Synthesis and purification of phosphorothioate ODNs for tissue culture experiments was performed as previously described[31,37]. Synthesis and purification of ODNs used for animal studies was performed as previously described[43].

Treatment of cells in culture with antisense oligonucleotides. Cells growing on 10-cm plates at a density of 50–75% confluency were treated with ODNs in the presence of cationic lipid (Lipofectin reagent, Gibco BRL) as previously described[31,32,37].

Northern blot and western blot analysis. For determination of mRNA levels by northern blot, total RNA was prepared from cells or tumors by the guanidinium isothiocyanate procedure[44] 24 h after initiation of ODN treatment for cell culture studies or at the indicated times for *in vivo* antitumor studies. Total RNA was isolated by centrifugation of the cell lysates or tumor extracts over a cesium chloride cushion[44]. Northern blot analysis was as previously described[44]. RNA was quantified and normalized to G3PDH mRNA levels using a Molecular Dynamics PhosphoImager, as described previously[37].

For determination of protein levels by western blot, cellular extracts were prepared using 250 µl of RIPA extraction buffer[44] per 10-cm dish. Typically, 25 µg of protein were then separated by electrophoresis on a 10% SDS–polyacrylamide mini-gel (BioRad). Once transferred, membranes were then treated at least 2 h with a monoclonal antibody that specifically recognizes C-*raf* kinase protein (Transduction Laboratories) at a dilution of 1:1,000 followed by incubation with 5 µCi of ^{125}I-labeled goat anti-mouse antibody (ICN Radiochemicals) for 1 h. Labeled proteins were visualized and quantified by PhosphorImage analysis (Molecular Dynamics).

Measurement of cell proliferation. Cells were seeded in 6-well tissue culture dishes at a density of 2×10^4 cells per well and allowed to attach to the plate overnight. The following day, cells were treated with the appropriate ODNs (as described above), at the concentrations described in the results section, for 4 h in the presence of DOTMA. Following treatment, the ODN-containing medium was replaced with the normal medium containing 10% FBS, and cells were incubated at 37 °C. Viable cell number was determined at the appropriate times following ODN treatment by direct counting using a hemacytometer. Each experimental condition was performed in triplicate, and the standard deviation for each group was determined. Cell viability was determined by trypan blue staining. Treated and untreated cells showed 98–100% viability after 24 h growth (with or without ODN).

Measurement of antisense oligonucleotide efficacy in nude mouse tumor models. Female BALB/c nude mice were obtained from Bomholtgaard, Copenhagen, Denmark, and used when 10–12 weeks old. The tumor cells used (A549, MDA-MD-231, T24) were implanted subcutaneously, and then serially passaged by a minimum of three consecutive transplantations before the start of treatment. Tumor fragments (approximately 25 mg) were implanted subcutaneously into the left flank of the animals with a 13-gauge trocar needle under Forene anesthesia (Abbott Laboratories, Basel, Switzerland). ODN treatments were initiated when the tumors reached a mean tumor volume of 100 mm³. ODNs (formulated in saline solution) were administered intravenously daily by bolus infusion into the tail vein at the indicated doses. The number of times ODN was administered within a given study varied depending on the experiment and is described for each experiment in the figure legends. Tumor volumes were monitored two or three times weekly and 24 h following the final treatment by measuring perpendicular diameters. Tumor volumes were calculated as described previously[41]. Each experimental condition included at least five animals per group. Average tumor volumes and standard deviations were calculated for each group and plotted. For determining C-*raf* kinase mRNA levels in tumors, tumor xenografts were prepared and treated with the appropriate ODNs at a concentration of 6.0 mg/kg, and tumors were removed at the indicated times following initiation of ODN treatment. Total RNA was prepared from excised tumors and analyzed by northern blot as described above. C-*raf* mRNA was quantified by PhosphorImage analysis and normalized to G3PDH mRNA levels. Average C-*raf* mRNA levels were plotted as a percentage of C-*raf* mRNA levels in saline-treated animals (percent control). Three animals per experimental condition were employed.

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APPENDIX C

Antisense Oligonucleotides as Therapeutic Agents

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Antisense oligonucleotides can block the expression of specific target genes involved in the development of human diseases. Therapeutic applications of antisense techniques are currently under investigation in many different fields. The use of antisense molecules to modify gene expression is variable in its efficacy and reliability, raising objections about their use as therapeutic agents. However, preliminary results of several clinical studies demonstrated the safety and to some extent the efficacy of antisense oligodeoxynucleotides (ODNs) in patients with malignant diseases. Clinical response was observed in some patients suffering from ovarian cancer who were treated with antisense targeted against the gene encoding for the protein kinase C-alpha. Some hematological diseases treated with antisense oligos targeted against the bcr/abl and the bcl2 mRNAs have shown promising clinical response. Antisense therapy has been useful in the treatment of cardiovascular disorders such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. Antisense oligonucleotides also have shown promise as antiviral agents. Several investigators are performing trials with oligonucleotides targeted against the human immunodeficiency virus-1 (HIV-1) and hepatitis viruses. Phosphorothioate ODNs now have reached phase I and II in clinical trials for the treatment of cancer and viral infections, so far demonstrating an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, based on a phosphorothioate oligonucleotide designed to inhibit the human cytomegalovirus (CMV), promises that some substantial successes can be reached with the antisense technique. J. Cell. Physiol. 181:251–257, 1999. © 1999 Wiley-Liss, Inc.

The use of oligonucleotides as selective inhibitors of gene expression offers a rational approach for the prevention and treatment of some gene-mediated disorders. In the antisense approach, oligonucleotides block the expression of specific target genes involved in the development of the pathological processes. Therapeutic applications of antisense technique currently are under investigation in many different fields, including oncology, hematopathology, cardiovascular diseases, and infectious diseases (Agrawal and Iyer, 1995; Wagner, 1995; Agrawal, 1996; Crooke and Bennet, 1996; Bradbury, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

Antisense oligodeoxynucleotides (ODNs) are short stretches of DNA (12–30 nucleotides) that are complementary to a target mRNA. The ODNs selectively hybridize to their complementary RNA by Watson-Crick base pairing rules. The translation of target mRNA is inhibited by an active and/or a passive mechanism when hybridization occurs between the ementary helices. Passive mechanism results from the hybridization between the mRNA and exogenous nucleotide sequence, which leads to duplex formation that prevents the ribosomal complex from reading the message (Fig. 1A). In the active mechanism, hybridization allows for binding of RNaseH, which destroys the RNA but leaves the DNA oligonucleotide intact to hybridize with yet another mRNA target (Fig. 1B; Wagner and Flanagan, 1997; Bradbury, 1997; Monia, 1997; Kronenwett and Haas, 1998b).

The concept of antisense technology is simple. However, the development of antisense oligonucleotides as broadly applicable therapeutic agents has been slow and difficult (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Bradbury, 1997; Wagner and Flanagan, 1997; Romano et al., 1998a).

SELECTION OF A SPECIFIC AND EFFECTIVE ANTISENSE MOLECULE

Selection of target sequence

The selection of an appropriate target sequence is the first step in the process of drug development. In

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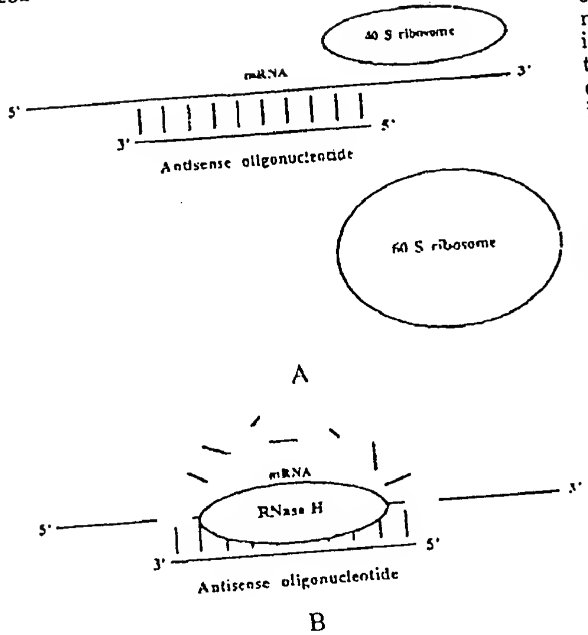

Fig. 1. Mechanism of action of antisense oligonucleotides. In the passive mechanism (A), the translation of target mRNA is inhibited by the hybridization between the mRNA and exogenous nucleotide sequence. This leads to duplex formation that prevents the ribosomal complex from reading along the message. In the active mechanism (B), the mRNA-ODN heteroduplex forms a substrate for RNase H, an enzyme that recognizes and selectively destroys the RNA portion of the mRNA-ODN hybrid.

fact, the hybridization between antisense oligos and the target sequence, which has a particular three-dimensional structure resulting from secondary and tertiary structures, depends on the accessibility of the target sequence. Only small stretches of mRNA sequence, devoid of interchain hybridization, are available for heteroduplex formation with DNA oligonucleotides which affects the activity of ODNs. For example, only one of 34 ODNs targeting human c-raf mRNA demonstrated potent antisense activity. Modeling of the secondary structure of the target mRNA by computer software can be used for target selection of antisense molecules. Such a method carefully considers the potential folding pattern of a chosen mRNA as derived from its particular nucleotide sequence. After determining the free energy of a given secondary structure, the most probable folding structures are indicated, showing open loops and bulges that are accessible for oligonucleotides for efficient hybridization (Stein and Krieg, 1994; Monia, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

Selection of chemical modifications

Cells contain a variety of exo and endonucleases that can degrade ODNs. A number of nucleotide and nucleoside modifications have made the oligonucleotide more resistant to nuclease digestion than the native ODNs that have phosphodiester linkages in their nucleotide backbone. Oligonucleotides that have been modified to enhance their nuclease resistance survive intact for longer times than unmodified oligonucleotides. A variety of oligonucleotide modifications have enhanced or conferred nuclease resistance, thus allowing oligos to reach their intracellular targets (Capaccioli et al., 1993; Gewirtz, 1993; Agrawal and Iyer, 1995; Galderisi et al., 1999). Phosphorothioates are one of the most frequent variants of ODNs. One of the oxygens in the phosphate backbone in these molecules is replaced by a sulfur atom (Fig. 2). Increased protection against cleavage by both exonucleases and endonucleases is the result of such chemical modification. Other modifications give rise to methylphosphonates, in which a methyl group is substituted for an oxygen of phosphate; other modifications are phosphoroamidates that show an amide linkage inserted instead of an external phosphate bridge; peptide nucleic acid, having the phosphate sugar backbone substituted by an alkylamide linkage (Fig. 2; Eckstein, 1983; Henry et al., 1997b; Flanagan, 1998; Galderisi et al., 1999). The oligonucleotides also may be "chimeric oligonucleotides." Chimeric oligonucleotides contain two or more chemically distinct regions. These molecules are designed to confer more than one beneficial property to ODNs, such as increased nuclease resistance, increased uptake into cells, or increased binding affinity for the RNA target. At present, phosphorothioate oligos are the most widely used molecules in cell cultures, animals, and humans (Agrawal and Iyer, 1995; Monia, 1997; Agrawal and Zhao, 1997; Shinozuka et al., 1997; Galderisi et al., 1999).

Cellular delivery of ODNs

The main problem in increasing the bioavailability of administered ODNs is the protection against cleavage. While the mechanism involved in the cellular ODN uptake still is not clear, there also is a great variation between different cell types with regard to their ability to internalize oligo molecules. A receptor-mediated endocytosis seems to play a main role in ODN uptake, followed by the release of ODNs from endocytotic vesicles into the cytoplasm (Loke et al., 1989; Iversen et al., 1992; Bennett et al., 1994; Beltinger et al., 1995; Kronenwett and Haas, 1998b).

The cellular internalization of ODNs is not efficient in in vitro models, hence, many techniques have been used to enhance ODN uptake. The most widely used method is based on cationic lipids. These molecules form complexes with the anionic nucleic acids and protect them against degradation. The macromolecular complexes have a positive charge at the surface, allowing binding to cell membrane, which is negatively charged. Following attachment to the membrane, the complexes are taken up via endocytosis. Additional improvement in oligo uptake can be anticipated. For example, efforts are being made to modify liposomal lipids by adding ligands of cellular receptors as well as antibodies directed against antigens expressed on the respective target cells (Bennet et al., 1992; Capaccioli et al., 1993; Stein and Krieg, 1994; Beltinger et al., 1995; Gokhale et al., 1997; Kronenwett and Haas, 1998b).

While direct administration of ODNs in vitro is not an effective delivery method, phosphorothioate ODNs

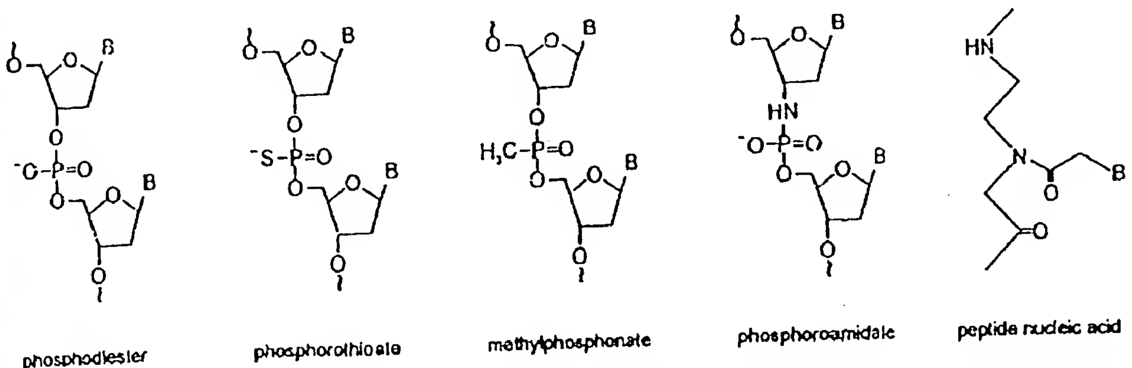

Fig. 2. Chemical structure of internucleotide linkages of unmodified (phosphodiester) and modified oligonucleotides. B, nucleotide bases.

administered intravenously without any delivery reagent to animal models showed effective and specific antisense inhibition. These surprising results helped revive antisense technology and encouraged researchers to move to clinical trials (Wagner, 1995; Crooke and Bennet, 1996; Geary et al., 1997).

Antisense oligonucleotide evaluation

The aim of antisense researchers is to show downregulation of a target gene in a sequence-specific manner, while control ODNs, which are oligonucleotides not complementary to the chosen target mRNA, should show little or no downregulation capability. However, several examples of nonsequence-specific effects have been seen with ODNs, particularly with the chemically modified molecules. Oligonucleotides, which are negatively charged, can interact with positively charged molecules. For example, ODNs can bind in a sequence-independent manner the gp120 protein of the human immunodeficiency virus-1 (HIV-1), bovine serum albumin, the receptor for platelet-derived growth factor, the receptor for basic fibroblast growth factor, and several other cellular proteins (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Crooke and Bennet, 1996).

Antisense side effects also could be due to sequence-specific interactions between ODNs and cellular proteins that can cause the so-called "sequence-dependent but nonantisense effect." For example, the presence of four contiguous guanosine residues in an ODN, the G quartet, can result in an antiproliferative effect regardless of the remaining sequence of the molecule (Crooke and Bennet, 1996; Stein and Krieg, 1994; Vaerman et al., 1995; Wagner, 1995).

ANTISENSE THERAPY

The idea of antisense-mediated gene inhibition therapy is as fascinating as other types of gene therapy (Romano et al., 1998a; Giordano et al., 1998). The following examples suggest that these compounds may have some therapeutic efficacy likely through a combination of antisense and nonsequence-dependent effects on gene function.

Pharmacokinetics

Several experiments assessing the pharmacokinetics and toxicology of ODNs have been performed in mice, rats, and monkeys (Cossum et al., 1993; Galbraith et al., 1994; Iversen et al., 1995; Zhang et al., 1995; Leeds et al., 1998). The pharmacokinetics were independent of the length as well as of the sequence of ODNs. When injected intravenously or intraperitoneally, the nucleic acids were excreted mainly in the urine within 24 h. However, detectable levels were found in most tissues except the brain for up to 48 h, with only 15–50% degradation for phosphorothioate ODNs . In monkeys and in phase I clinical trials, dose-dependent hypotension, complement activation, and transient prolongation of thromboplastin time were observed as side effects. Preliminary results of other clinical studies demonstrated the safety and, to some extent, the efficacy of antisense ODNs in patients with malignant diseases (Iversen et al., 1995; Glover et al., 1997; Henry et al., 1997a; Raynaud et al., 1997; Sereni et al., 1999).

Antisense clinical trials for cancer treatment

A major signal transduction pathway involving the enzyme protein kinase C (PKC) has a critical influence on cell proliferation and differentiation (Liu and Heckman, 1998). An increased expression of PKC-alpha is found in many human cancers including those of the breast and colon and in brain tumors. Inhibition of human PKC-alpha gene expression has occurred with antisense ODNs both in vitro and in vivo (Dean et al., 1996; Zhang et al., 1997). A phosphorothioate ODN, directed against the 3'-untranslated region of PKC-alpha, has been tested by ISIS Pharmaceutical (Carlsbad, CA) and Novartis (Basel, Switzerland) in some human tumor cell lines grown in athymic mice. This oligo, named ISIS3521, was administered intravenously once a day for 14 days and showed a noticeable tumor growth decrease in T-24 bladder carcinoma, in A-549 non-small cell lung carcinoma, and in Colo 205 colon carcinoma xenograft models with a 50% inhibitory dose between 60 and 600 μg/kg per day. After this success, ODN entered a phase I clinical trial. Clinical responses were observed in 3 of 17 treated patients, all having ovarian cancer (McGraw et al., 1997; Flanagan, 1998).

The c-raf gene codes for a highly conserved serine-threonine-specific protein kinase (Magnuson et al., 1994; Kerkhoff and Rapp, 1998; Yuryev and Wennagle, 1998). Certain abnormal proliferative conditions are associated with raf expression and therefore are believed to be responsive to inhibition of raf expression (Worland et al., 1990). Examples of abnormal proliferative conditions are hyperproliferative disorders such as cancers, hyperplasias, pulmonary fibrosis, and angiogenesis (Nakatsu et al., 1986; Pfeifer et al., 1989; Naumann et al., 1997).

A phosphorothioate ODN named ISIS5132, which is complementary to c-raf mRNA, has shown a strong sequence-specific inhibition of c-raf gene expression in some subcutaneously implanted human tumor cell lines in nude mice. Subsequently, phase I clinical trials have demonstrated the safety of this ODN. Furthermore, several patients with breast, prostate, and colon cancers showed promising clinical response. Based on this data, phase II clinical trials were initiated in 1998 (Monia et al., 1996; Henry et al., 1997b; McGraw et al., 1997; Monia, 1997; Flanagan, 1998; Monteith et al., 1998).

The ODNs may also be useful for ex vivo bone marrow purging, a method used for treatment of patients suffering from leukemias and lymphomas. Large amounts of bone marrow can be surgically extracted from patients and stored in vitro, while the patients receive conventional treatment. Following relapse, the patients can be rescued by reinfusion of their own bone marrow cells that have been "purged" of residual malignant cells employing ODNs targeted against altered gene expression associated with the leukemias and/or lymphomas (De Fabritiis et al., 1998).

Bcr/abl mRNA is the product of a neo-gene created by a reciprocal translocation involving the c-abl and the bcr genes. The expression of the bcr/abl oncogene is involved in the pathogenesis of chronic myelogenous leukemia (CML). Some clinical experience with bcr/abl targeted antisense ODNs in CML has been reported (Vaerman et al., 1995; Skorski et al., 1997; Kronenwett and Haas, 1998a). De Fabritiis and colleagues (1998) treated a patient with CML in an accelerated phase with autologous bone marrow transplantation. Before reinfusion, cells were purged in vitro with a 26-mer phosphorothioate ODN targeted against bcr/abl mRNA. The patient was reported to be in complete hematological remission (Gewirtz, 1993; De Fabritiis et al., 1998).

The expression of the bcl2 gene, which is involved in the apoptosis pathway, is overregulated in most non-Hodgkin lymphomas (Reed et al., 1990; Tsurusawa et al., 1998; Reed, 1998). Genta (San Diego, CA) has developed an antisense ODN targeted against bcl2 mRNA. This oligo results in a complete remission in nude mice inoculated with human follicular lymphoma cells. Based on these results, phase I trials have been initiated (Raynaud et al., 1997; Webb et al., 1997; Flanagan, 1998; Kronenwett and Haas, 1998b; Bloem and Lockhorst, 1999; Chaudhary et al., 1999).

Antisense ODN as potential drugs in other human diseases

Antisense therapy is emerging as a potential agent for the treatment of cardiovascular diseases such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. The local transfer of antisense molecules into the vascular wall offers a promising alternative for the treatment of atherosclerosis-related diseases at the cellular and molecular levels. Blood vessels are among the easiest targets for this gene therapy technique because in such conditions as postangioplasty restenosis, only a transient inhibition of target gene expression is required (Shi et al., 1994; Laitinen and Yla-Herttuala, 1998).

Coronary balloon angioplasty is a procedure in which a catheter bearing an inflatable distal balloon is inserted into the arterial lumen and expanded. The method is used to open stenotic regions in vessels closed by arterial plaques and fatty deposits. This technique is used often for patients suffering from atherosclerosis. Smooth muscle cell (SMC) proliferation of the vascular wall is a normal response to several pathophysiological stimuli, including those associated with procedures for mechanically opening stenoses. If the proliferation is extensive, restenosis could follow the procedure. In particular, as many as 50% of the patients undergoing successful coronary angioplasty can develop recurrent coronary artery obstructions. Several different classes of pharmacological agents have been employed to inhibit SMC proliferation but as yet, unsuccessfully.

One approach is to inhibit mitogens that act on the cell surface of SMCs. The c-myc gene product is encoded by an immediate-early response gene, the expression of which can be induced by various mitogens. C-myc expression is involved in the signal transduction pathways leading to cell division. Studies have demonstrated that proliferating cells have higher levels of c-myc mRNA and protein than do quiescent cells (Paggi et al., 1996; Nesbit et al., 1999).

Several investigators have demonstrated the in vitro growth-inhibitory effect of antisense oligomers targeting the c-myc proto-oncogene in human SMCs (Bennett et al., 1994; Shi et al., 1994). These in vitro studies provided the rationale for assessing c-myc antisense oligomers in the prevention of neointima in vivo. For this purpose, antisense oligomers targeted against c-myc mRNA were delivered into balloon-denuded porcine coronary arteries. Despite rapid plasma clearance following local delivery, oligomers persisted at the site of injection for at least 3 days, exceeding by severalfold their concentration in peripheral organs. The morphometric analyses, carried out 1 month after transcatheter c-myc antisense oligomer administration, showed a significant reduction in maximal neointimal thickness in the antisense-treated group compared with controls. These changes in vascular remodeling following denuding injury resulted in an increase in the residual lumen in the antisense-treated animals. Since c-myc antisense oligomers reduced the formation of neointima in denuded coronary arteries, a potential therapeutic use for the prevention of coronary restenosis can be hypothesized (Shi et al., 1994; Mannion et al., 1998).

The use of antisense oligonucleotides has also emerged as a powerful new approach as antiviral agents (Selvam et al., 1996; Caselmann et al., 1997; Lima et al., 1997; Wagner and Flanagan, 1997; Veal et al., 1998). In fact, the initial therapeutic applications of ODN were supposed to be as an antiviral agent. Ste-

phenson and Zamecnik (1978) disclosed antisense oligonucleotides as inhibitors of Rous sarcoma virus replication in chicken fibroblasts.

HIV is responsible for the disease that has come to be known as acquired immune deficiency syndrome (AIDS). The HIV genome tends to mutate at a high rate, causing great genetic variation between strains of the virus and between virus particles of a single infected individual. Therapeutic agents currently used in the treatment of AIDS often cause severe side effects that preclude their use in many patients (Zhang et al., 1995; Junker et al., 1997; Romano et al., 1998b; Sereni et al., 1999).

One method for inhibiting specific gene expression that is believed to have promise is the antisense approach. Inhibition of viral gene expression and replication can be efficiently achieved by targeting the conserved sites of the viral RNAs that signal the synthesis of conserved HIV proteins, particularly the p24 core antigen protein. Some research groups have synthesized 20 mer/15 mer sequences targeted against the p24 core protein region of HIV. Initial clinical trials are based on ODN systemic administration (intravenously). Dosages that can be used for systemic administration preferably range from about 0.01 to 50 mg/kg administered once or twice per day. Evaluations of ODN activity are under examination (Zhang et al., 1995; Junker et al., 1997; Sereni et al., 1999).

Chronic infection with the hepatitis B virus (HBV) is a major health problem worldwide. The only established treatment is interferon with an efficacy of only 30–40% in highly selected patients. The discovery of animal viruses closely related to the HBV has contributed to active research on antiviral therapy of chronic HBV infection. The animal model tested and described by several authors are Peking ducks infected with the duck HBV (DHBV; Shinozuka et al., 1997; Soni et al., 1998; Xin and Wang, 1998). Molecular therapeutic strategies are based on antisense ODNs directed against the 5′-region of the preS gene of DHBV that inhibited viral replication and gene expression in vitro in primary duck hepatocytes. The in vivo studies showed that intravenous injection of antisense ODNs entrapped within liposomes enhances delivery of the ODNs to the liver and inhibits DHBV replication. Serum DHBV DNA levels fall rapidly, with a corresponding decrease in intrahepatic viral replicative intermediates at the end of the 5-day study period. These results demonstrate a potential clinical use for antisense DNA as antiviral therapeutic agents (Caselmann et al., 1997; Lima et al., 1997; Offensperger et al., 1998; Soni et al., 1998).

THE FIRST ANTISENSE-BASED DRUG

Vitravene is the first in a class of novel therapeutic agents based on an antisense mechanism that has been approved for marketing in the United States. Vitravene is indicated for the local treatment of cytomegalovirus (CMV) retinitis in patients with AIDS who are intolerant to or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments (from www.vitravene.com). Vitravene consists of a phosphorothioate oligonucleotide designed to inhibit human CMV replication by an antisense mechanism. It has been shown in vitro to inhibit replication of human CMV with a greater potency than either ganciclovir or foscarnet. It does not interfere with the antiviral activity of the anti-HIV drugs AZT and dideoxycitidine and it can be additive to the use of ganciclovir and foscarnet. Vitravene was equally potent against 21 independent clinical human CMV isolates, including several that were resistant to ganciclovir, foscarnet, and/or cidofovir (from www.vitravene.com).

FUTURE PROSPECTS

The use of antisense to modify gene expression is variable in both its efficacy and reliability, which caused objections about its use as a therapeutic agent. Most of these concerns can be overcome by the development of a new generation of antisense molecules with improved target specificity and enhanced delivery to the target cells. However, one concept must be borne in mind, an oligonucleotide need not be exclusively complementary to its target nucleic acid sequence to be specific. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule sufficient to cause a loss of function. There is a sufficient degree of complementarity to avoid nonspecific binding of the oligonucleotide to nontarget sequences (Stein and Krieg, 1994; Stein, 1995; Monia, 1997).

Antisense ODNs already have shown their effectiveness in several preclinical studies. Phosphorothioate ODNs have reached phase I and II in clinical trials for the treatment of cancer and viral infections and have demonstrated an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, which is based on an antisense mechanism and is commercially available in the United States, has shown that some substantial successes can be reached with the antisense technique.

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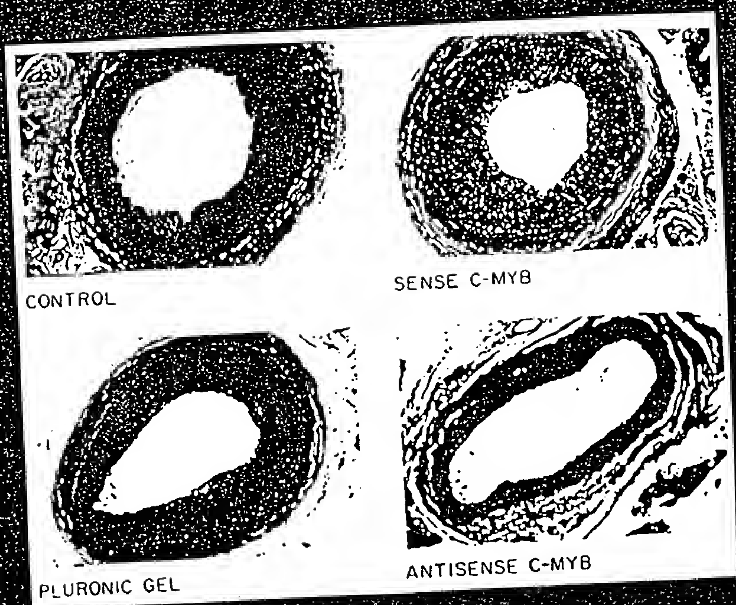
APPENDIX D

METHODS IN MOLECULAR MEDICINE™

Antisense Therapeutics

Edited by
Sudhir Agrawal



Humana Press

METHODS IN MOLECULAR MEDICINE™

Antisense Therapeutics

Edited by

Sudhir Agrawal

Hybridon, Inc., Worcester, MA

Humana Press Totowa, New Jersey

Preface

Antisense nucleic acids (antisense therapeutics) have attracted much interest as a novel class of therapeutic agents for the treatment of viral infections, cancers, and genetic disorders because of their ability to inhibit the expression of disease-associated genes. Antisense therapeutics inhibit gene expression in a sequence-specific manner through hybridizing to the target gene through Watson-Crick base pairing. Antisense therapeutics have two characteristics required for successful drug design—specificity and affinity. Theoretically, an antisense oligonucleotide comprising 17 nucleotides is unique for its target in the human genome and its affinity for the target is much higher than the 37°C body temperature.

The laboratory of Paul Zamecnik published the first report of antisense oligonucleotides in 1978 in two papers in the Proceedings of the National Academy of Sciences. He and his colleague Mary Stephenson showed that a synthetic oligonucleotide (13-mer) complementary to the Rous sarcoma virus genome inhibited the viral replication when added exogenously to the infected cell culture. Based on these findings, he remarked in his paper on the general chemotherapeutic potential of this observation. I am very fortunate to have worked in Paul Zamecnik's laboratory and have learned a great deal from him about various aspects of antisense therapeutics.

Research efforts exploring the possibility of using oligonucleotides as therapeutic agents started in the mid-1980s. The focus shifted to identification of oligonucleotide analogs that have specificity and affinity for the targeted gene and can be administered locally or systemically, are stable in vivo, and are safe in animals. These analogs thereby provide a wide therapeutic index. Enormous progress has been made in the last 10 years: Many analogs of oligonucleotides have been synthesized and studied as antisense agents. The most widely studied analog of oligonucleotides is phosphorothioate. The results of preclinical studies using oligodeoxynucleotide phosphorothioates have shown that antisense oligonucleotides have good biological activity, pharmacology, pharmacokinetics, and safety both in vitro and in vivo, and they are currently being evaluated in human clinical trials for the treatment of viral infections and cancers.

for prevention of development of escape mutants. Whereas escape mutants appeared after 20 d treatment of chronically infected Molt-3 cells with an antisense phosphorothioate oligomer pinpointing a splice acceptor site, continued inhibition without escape over an 84-d experimental period occurred when *rev1–28* or *gag-28* were the targets.

A second example is the use of antisense oligomers to inhibit influenza viral replication *(62)*. At 10 μM concentration, replication of influenza C virus was inhibited 90% in tissue cultures of MDCK cells by a sense oligophosphorothioate targeted against the replicase gene of the negatively stranded virus. In 10-d-old embryonated chick eggs, phosphorothioate oligomer injection also induced marked inhibition of virus production *(63)*.

Another example is inhibition of replication of *Plasmodium falciparum* malaria by a phosphorothioate oligodeoxynucleotide targeted against the dihydrofolate reductase-thymidylate synthase gene of the parasite *(64)*. This enzyme is essential as a donor of a methyl group in the conversion of deoxyuridine monophosphate to thymidine monophosphate in the parasite, which must synthesize its own pyrimidines, being unable to use exogenous thymidine for synthesis of DNA. The adult erythrocyte is one of the rare eukaryotic cells that oligodeoxynucleotides do not penetrate. Fortunately, however, when a malarial parasite pushes its way into a red cell, it creates a permeabilized erythrocyte membrane plus a parasitophorous duct *(65)*, through either or both of which the oligodeoxynucleotide reaches the parasite inside its protective erythrocyte envelope. A fluorescently labeled oligodeoxynucleotide lights up a circular area inside an erythrocyte in which the *P. falciparum* parasite resides, surrounded by its own membrane, whereas the uninfected red cells fail to show evidence of cell entry *(66)*. The above-mentioned antisense oligomer shows a sequence-specific ID_{50} for replication of the parasite at 2–5 × $10^{-8}M$ concentration *(67)*.

Thus, in summary, the synthetic antisense oligonucleotide technology has potential application to human diseases and displays promising results in cell-free systems, tissue cultures, and animal models. It is also at early trial points *(68,69)* in human testing against HIV, leukemia, Herpes virus, and other diseases, whose outcome will remain for the future. The current status of these varied approaches is presented in later chapters in this book.

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APPENDIX E

Review

Biologicals & Immunologicals

Patent strategies in the antisense oligonucleotide based therapeutic approach

Anne Craig, Darlene Vanstone & Sudhir Agrawal

Antisense oligonucleotides represent a new approach to therapeutic treatment of a variety of diseases. Due to unique aspects of this new approach, the opportunity to protect inventions *via* patenting is potentially greater than for small molecule pharmaceutical products. Strategies for protecting antisense technology include developing a multiple layered patent portfolio covering a range of compositions of matter having improved pharmacological properties. Such patentable new compositions of matter arise from exploitation of pharmacological properties discovered in preclinical and clinical trials, and from new synthetic and manufacturing processes, which provide new chemical entities. In addition, advances in manufacturing technology reduce the cost of antisense oligonucleotides and are independently patentable. In this article, the general strategic approach to patenting antisense technology will be presented, together with illustrative examples of the implementation of such a strategy. For this purpose, primarily numerous illustrations will be taken from selected patents from the patent portfolio of Hybridon, Inc., and relevent literature with which the authors have the greatest familiarity.

Keywords: Antisense oligonucleotides, composition of matter, formulations, oligonucleotide manufacture, oligonucleotide modifications, RNase H, 2'-O-substituted ribonucleoside

Exp. Opin. Ther. Patents (1997) 7(10):1175-1182

1. Introduction

The antisense therapeutic approach is based upon the recognition that inappropriate gene expression is responsible for most disease states. Such inappropriate gene expression may be in the form of a gene from a virus or a pathogen being expressed in a diseased cell or organism, as in the case of infectious diseases. Alternatively, it may be in the form of a cellular gene being expressed abnormally, either in a mutated form or in a temporally or spatially incorrect manner, such as in cancer. The nature of the disease is dictated by the particular gene that is being inappropriately expressed. In turn, the particular gene is defined by the sequence of its nucleotides. In the antisense therapeutic approach, a short region of nucleotides, or an oligonucleotide, which has a sequence that is complementary to the inappropriately expressed gene is allowed to hybridise to the transcript of such gene (i.e., mRNA), thereby preventing its expression [1,2]. An oligonucleotide can interrupt the process of genetic expression at various stages, as shown in Figure 1. The interaction between oligonucleotide and target mRNA is by Watson-Crick base pairing (called hybrid-

Figure 1: Mechanism of action of oligonucleotides. Oligonucleotides may interfere in RNA processing and translation at various stages, e.g., transcription, splicing of pre-mRNA, translocation of mRNA, ribosome assembly and ribosome migration.

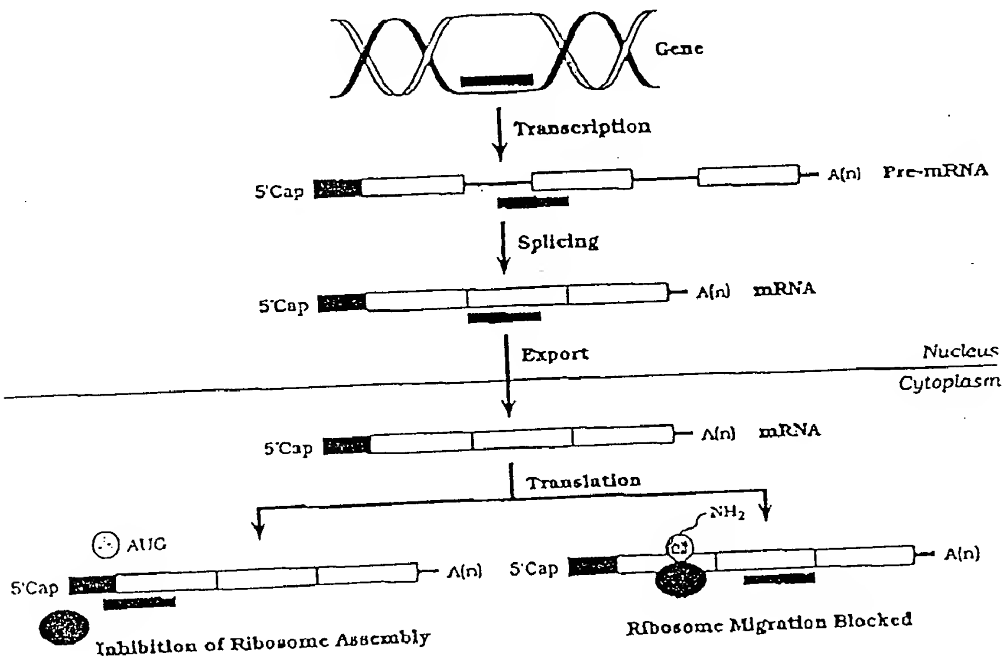


Figure 2: Interaction inbetween antisense oligonucleotide and target RNA or single-stranded DNA is by Watson-Crick base pairing between heterocyclic bases, a phenomenon called hybridization. In hybridization, adenine hydrogen bonds with thymine and guananine hydrogen bonds with cytosine.

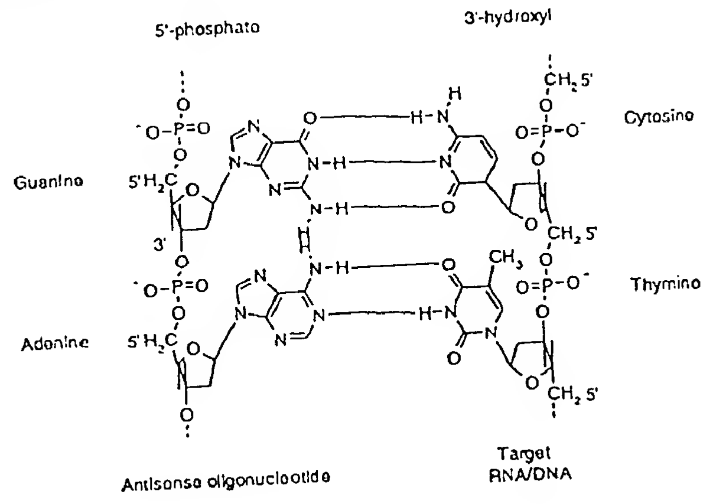


Figure 3: Graphic representation of duplex of antisense oligonucleotide and target RNA. There are two processes by which antisense oligonucleotide may inhibit mRNA processing/translation. In first process, antisense oligonucleotide may inhibit ribosome assembly or migration by physical blockage (A). In the second process duplex of antisense oligonucleotide and RNA becomes a substrate for RNase H (an endogenous enzyme), which then cleaves the RNA at the site of duplex, thereby permanently destroying the RNA.

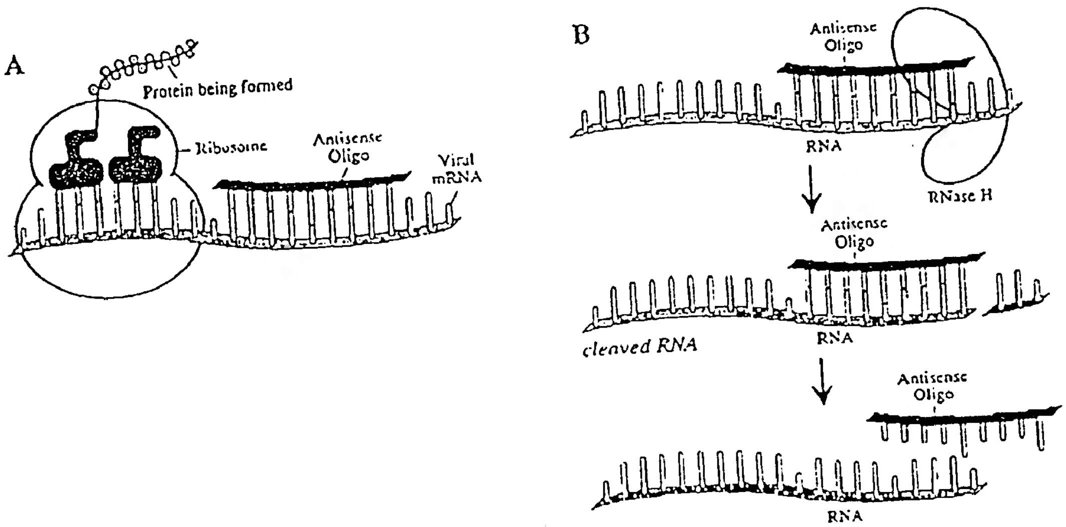
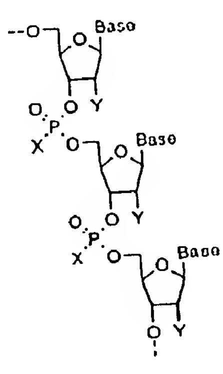


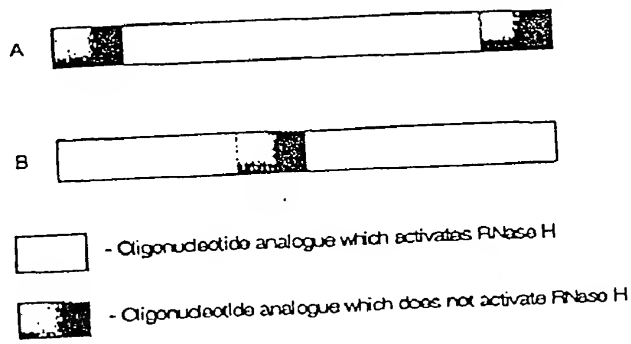
Figure 4: Structures of natural oligodeoxyribonucleotide ($X = O^-$, $Y = H$) and oligoribonucleotides ($X = O^-$, $Y = OH$). To improve the stability, number of analogs have been synthesized and studied, the two most studied analogs are phosphorothioate ($X = S^-$, $Y = H$) and methylphosphonate ($X = CH_3$, $Y = H$). Similarly number of oligoribonucleotides analogs have been sudied including phosphorothioate ($X = S^-$, $Y = OH$), or containing various 2'-substituted analogues including 2'-O-methylribonucleosides ($X = S^-$ or O^-, $Y = OCH_3$).



isation) which provides both specificity and affinity, the two important parameters for drug design (**Figure** 2). Following hybridisation, the oligonucleotide may interfere in the translation process by physically blocking ribosome assembly or migration and/or by excising the mRNA with the help of RNase H, an enzyme which recognises duplex of oligodeoxynucleotide and mRNA and excises the RNA strand at the duplex site (**Figure** 3) [3-9].

The disease treating specificity of the oligonucleotide is thus provided by its nucleotide sequence. More accurately, this specificity is provided by the order of the heterocyclic bases or nucleotides and their affinity to the target. Thus, the linkages of nucleotides or the oligonucleotide's internucleotide linkages (referred to herein as the 'backbone' of the oligonucleotide) may be modified to alter its other characteristics, such as its affinity to mRNA, RNase H activity, nuclease stability, bioavailability, pharmacokinetics and toxicity. A number of oligonucleotide analogues, both oligodeoxy as well as oligoribonucleotides, have been studied as antisense agents (**Figure 4**) [3-9]. Once such a modification is found to confer a favourable characteristic, it can then be used in oligonucleotides having different sequences of nucleosides and, thus, provide utility for the treatment of other diseases. This is in sharp contrast

Figure 5: Graphic representation of oligonucleotide which contain segment of oligonucleotides which activates RNase H and other segment which does not activate RNase H (dark shaded), however can be used to modulate metabolic stability and pharmacological properties of oligonucleotides in use. Oligonucleotide analogues which are known to activate RNase H are phosphodiester and phosphorothioate oligodeoxynucleotides. The two oligonucleotide designs are represented. [A] in which oligodeoxynucleoside phosphorothioate flanked by analogues of oligodeoxy- or oligoribonucleoside and in second [B] which contain segments of oligodeoxynucleoside phosphorothioate in flanking region and analogues of oligodeoxynucleoside or oligoribonucleoside in the center of the oligonucleotide.



to traditional small molecule drugs, for which such a useful modification is not normally transferable to other chemical entities useful for the treatment of other diseases.

2. Strategic opportunities presented by antisense technology

The severability of the type of oligonucleotide backbone from the sequence of nucleosides to be employed provides a unique opportunity for independent but complementary types of patent protection for therapeutic oligonucleotides. This opportunity leads to an overlapping or layered strategy for patent protection. According to this strategy, a first 'layer' of patent protection is provided by the chemical nature of the oligonucleotide backbone. This type of protection is independent of the sequence of nucleosides, and is thus independent of the nature of the disease to be treated. A second layer of protection resides in the particular sequence of nucleosides of the therapeutic oligonucleotide. This type of protection is complementary to the first type because it is specific to the gene target, but independent of the type of backbone modification. A marriage of a particular backbone modification and a particular sequence of nucleosides produces a distinct therapeutic new chemical entity, which may itself be protected by an additional layer of patent protection.

3. Patenting of backbone modifications

The natural oligonucleotide is comprised of repeating units of phosphodiester bonds in position between the 5'-hydroxyl of one nucleoside and the 3'-hydroxyl of the neighbouring nucleoside, and the sugars which comprise each nucleoside include deoxyribose in the case of DNA and ribose in the case of RNA (**Figure 4**). The first chemical modifications of oligonucleotide backbones arose from efforts to replace a non-bridging oxygen from the phosphodiester bond with a different substituent. Among the earliest of these first generation modifications were oligonucleoside phosphorothioates [3-9] and oligonucleoside methylphosphonates [3-9], in which a non-bridging oxygen of each phosphodiester bond is replaced respectively with a sulfur atom or methyl group. These modified oligonucleotides were more stable to nuclease digestion than natural oligonucleotides. Although first generation oligonucleoside methylphosphonates have not yet been successfully developed as clinical candidates (perhaps due to their low affinity to RNA and inability to activate RNase H) numerous oligonucleoside phosphorothioates are now in clinical trials [8,9]. Patents were granted for these early modifications. For example, a number of patents related to first generation oligonucleoside methylphosphonates were issued to Johns-Hopkins University and subsequently exclusively licensed to Genta Incorporated [101-105]. Similarly, patents were issued to the National Institutes of Health (NIH) for oligonucleoside phosphorothioates [106-108].

Later backbone modifications arose from the recognition that oligonucleotides could be organised into different regions, each of which contains a different type of chemical modification. For example, 'chimeric' oligonucleotides were developed which incorporated regions of oligonucleoside phosphorothioate or phosphodiester together with regions of oligonucleoside

alkylphosphonate or phosphoramidate regions (**Figure 5**) [10]. These second generation oligonucleotides provided even more improved stability, while retaining the ability to destroy target RNA through an RNase H-mediated mechanism. Several patents on these chimeric oligonucleotides and their use have been issued to the Worcester Foundation for Biomedical Research and exclusively licensed to Hybridon [108-111].

Yet another second generation backbone modification utilises a combination of phosphorothioate internucleoside linkages together with a central region of deoxynucleosides flanked by regions of 2'-O-methyl ribonucleosides (**Figure 5**) [11]. This molecule, referred to as a 'hybrid' oligonucleotide, appears to be the first clinically significant second generation oligonucleotide and is presently in clinical trials for the treatment of cytomegalovirus infection. In addition, this hybrid oligonucleotide modification is shown to have oral bioavailability [12], a patented phenomenon [113]. A patent on hybrid oligonucleotides as a composition of matter was issued to the Worcester Foundation for Biomedical Research, and exclusively licensed to Hybridon [114].

Third generation backbone modifications have been developed as a result of two very distinct approaches. In the first approach, observations from *in vivo* clinical and preclinical studies have been exploited to produce oligonucleotides having improved pharmacodynamic properties. For example, experimentation with hybrid oligonucleotides *in vivo* resulted in the observation that by reversing the regions, in which deoxynucleosides regions flank a central 2'-O-substituted ribonucleoside region, produced an oligonucleotide having a greatly reduced side-effect profile. Additionally, these 'inverted' hybrid oligonucleotides were as stable to nuclease digestion as phosphorothioate oligodeoxynucleotides [13]. A patent has been issued which covers this class of oligonucleotides [115].

The second approach to developing new third generation backbone modifications arose from the invention of new manufacturing processes. For example, the invention of an oligonucleotide synthesis process using a new base protecting group, N-pent-4-enoyl, has opened a new field of modified oligonucleotide backbones due to its ability to be removed under mild conditions [14,15]. Thus, chimeric oligonucleotides containing methylphosphotriester [16] or primary phosphoramidate [17] internucleoside linkages can now be made. Such new chimeric molecules have the potential to be very useful as antisense therapeutics. Hybridon's issued patent includes claims to the novel synthesis process and the novel synthons required to make these modified oligonucleotides [116].

These illustrations demonstrate that backbone modification provides a powerful source of patent protection for antisense oligonucleotides. This type of protection is especially important because it applies to any oligonucleotide having the proprietary backbone configuration, irrespective of its nucleoside sequence or the disease it is designed to treat. Numerous other modified oligonucleotide backbones have been described in the patent literature, although clinical development of such modifications has not yet been reported [9] [e.g. 117-119].

4. Patenting of oligonucleotide sequences

Patenting of oligonucleotides based upon their nucleoside sequence provides a separate and independent, but complementary layer of patent protection. Such patents may be obtained based upon two different reasons. Broad nucleoside sequence patents may be obtained when the causal relationship of expression of a particular gene, for example, with a particular disease has not been established and the patentee is the first to prove that such a relationship exists. In such situations, patents may be obtained for any oligonucleotide which has a nucleotide sequence complementary to the gene, provided that such oligonucleotide inhibits the expression of the gene. For example, researchers at Hybridon and Massachusetts General Hospital demonstrated that overexpression of beta amyloid protein caused cultured neural cells to undergo morphological changes similar to those observed in the brains of Alzheimer's disease victims, and that treatment of such altered cells with antisense oligonucleotides reversed such morphological changes. A patent was issued jointly to MGH and Hybridon, broadly claiming modified oligonucleotides complementary to the beta amyloid gene [120]. Patents of this type are of considerable value, because they can cover a host of potential chemical modifications, as long as the oligonucleotide targets the gene of interest.

However, whether or not the causal relationship of a gene to a particular disease is previously known or discovered by the patentee, it remains possible to obtain patent protection for particularly active antisense oligonucleotides based upon their nucleobase sequence. The patentability of such oligonucleotides resides in the unpredictability of which nucleoside sequence will produce the most effective antisense oligonucleotide. For example, Hybridon has been issued several patents claiming a variety of particular antisense oligonucleotides which are highly active as inhibitors of the expression of the vascular endothelial growth factor (VEGF) gene [121-123], which has now been implicated in diabetic retinopathy, retinopathy of

prematurity, age-related macular degeneration and a variety of metastatic cancers. While this type of protection covers only particularly active oligonucleotides, it is still a composition of matter patent, and thus provides its protection regardless of the type of disease which is to be treated. This can be of particular value for genes which are implicated in multiple diseases, such as VEGF.

5. Patenting oligonucleotide drug new chemical entities

An additional level of patent protection is available for a particular oligonucleotide drug new chemical entity in which both the type of backbone modification and the nucleobase sequence are defined [18]. This type of protection is reminiscent of traditional pharmaceutical patents covering new chemical entities. As second and third generation backbone modifications advance through clinical trials, new composition of matter patents on new chemical entities having these backbone modifications and defined nucleobase sequences may be obtained.

6. Patenting new oligonucleotide formulations

Yet another type of composition of matter protection available for antisense oligonucleotides arises from the manner in which the oligonucleotides are formulated. Formulation can provide benefits such as improved cellular uptake of oligonucleotides and improved side-effect profile [19,20]. For example, Hybridon has received patents covering the formulation of antisense oligonucleotides with cyclodextrins [124,125]. An advantage provided by this type of protection is that it can be independent of both the oligonucleotide sequence and the type of backbone modification present in the oligonucleotide.

7. Putting it together: composition of matter patents

The various and complementary layers of patent protection for antisense oligonucleotides obviously provide secure protection for antisense oligonucleotide drugs. Each layer comprises an independently patentable claim, with its own right of enforcement and its own presumption of validity under the patent laws. In addition, the nature of their discovery can serve to effectively extend the life of patent protection for specific oligonucleotide drugs. For example, early broad patents on backbone modifications may provide the first-in-time protection for an antisense oligonucleotide drug. Once the nucleotide sequence of the drug has been optimised, however, this can provide the basis for a later-filed patent application, and thus a patent which expires later. Similarly, when the perfect marriage between nucleotide sequence and backbone modification is subsequently discovered, patent life can be further extended. Finally, once the best formulation for the particular oligonucleotide drug is discovered, still further extension of patent protection becomes available.

8. Patenting oligonucleotide manufacturing

The unique nature of antisense oligonucleotides as drugs also provides opportunities for unusually strong manufacturing patents. This is, in part, because, unlike the case for traditional small molecule drugs, many manufacturing improvements for antisense oligonucleotides are applicable to a wide variety of different antisense oligonucleotide drugs. This advantage arises once again from the severability of backbone modification and nucleoside sequence.

Innovation in manufacturing technology provides three principle benefits:

- it provides antisense oligonucleotides in ever purer form
- it drives down the cost of manufacturing oligonucleotides
- it allows new classes of antisense oligonucleotides to be created

Each of these benefits can be enhanced through patent protection. Such protection can include patents covering novel synthons, catalysts for coupling, solution or solid phase synthesis processes, deprotection conditions and purification procedures.

Improved purity of oligonucleotides can be achieved by improving purification methodologies. Improved purification procedures are patentable. For example, Hybridon exclusively licenses two patents on improved purification processes from the Worcester Foundation for Biomedical Research [126,127]. These processes can be used with oligonucleotides having any type of backbone modification and any nucleoside sequence.

Lowering oligonucleotide manufacturing costs can be achieved by decreasing the time required for synthesis, improving the efficiency of synthesis and designing around older patents in the field. Each of these achievements can result in patent protection. For example, use of nucleoside synthons having the N-pent-4-enoyl base protecting group allows oligonucleotides

to be synthesised, and deprotected rapidly under milder conditions [16,17,21]. Hybridon has obtained a patent on these synthons [116]. In another example, the use of a new passivated organic solid support for oligonucleotide synthesis improves the efficiency of oligonucleotide synthesis. Hybridon also obtained a patent on this type of support [128]. Finally, a number of older patents cover certain types of nucleoside synthons, synthesis processes and sulfurizing reagents [129-135]. The development of new synthons, processes and sulfurising reagents which fall outside the scope of these patents will further reduce raw materials costs for oligonucleotide synthesis. These new synthons, processes and sulfurising reagents are likely to be independently patentable. Independent patenting of improvements in manufacturing technology is advantageous because it allows the company which is developing the technology to reduce its manufacturing costs without allowing its competitors to take unauthorised advantage of these benefits.

Manufacturing technology advances also open the door to the development of new classes of advanced antisense oligonucleotides. As mentioned earlier, the development of the N-pent-4-enoyl moiety as a base protecting group enabled the production of new classes of oligonucleotides containing methylphosphotriester or primary amidate internucleoside linkages. The patent obtained on this protecting group and its use in oligonucleotide synthesis prevents unauthorised synthesis of such compounds through the only presently known approaches.

A further benefit provided by manufacturing patents is that they provide protection in the United States against importation, use or sale of any antisense oligonucleotide composition which is manufactured outside the United States using the patented process. This prevents third parties from taking advantage of the absence of a process patent in a foreign country to produce an oligonucleotide for the US market. In addition, it provides this protection whether or not a composition of matter patent is obtained in the United States.

9. Expert opinion

Antisense oligonucleotide drugs provide unique opportunities for patent protection due to the severability of their backbone modifications and their nucleotide sequences. In the case of composition of matter patents, independent and complementary patents provide layers of patent protection which provide considerable strength of protection as well as the ability to extend the term of available protection. In the case of manufacturing patents, the opportunity arises to increase purity and develop new classes of compounds while simultaneously reducing costs, without providing competitors with the ability to exploit the new discoveries without authorisation. Taken together, relatively few strategically important patents are able to protect core technology and promote innovation in this exciting new field of antisense drug discovery.

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